PLANT PHYSIOLOGY

VOLUME 34 JULY, 1959 NUMBER 4

# STUDIES ON AUXIN-MEMBRANE PERMEABILITY RELATIONS IN FRUIT AND LEAF TISSUES [1]

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In a previous paper (22) I reported on the relationship between auxin and membrane permeability in bean endocarp tissue and in abscission phenomena in Coleus. Segments of the pods of Kentucky Wonder pole beans, prepared in the manner described for the Wehnelt bean test (4), remain rigid and plump up to 17 days when stored in the dark at 25° C in solutions of 4 to 50 ppm naphthalene acetic acid (NAA) or indoleacetic acid (IAA). In contrast, control segments in distilled water become soft and flaccid in 3 days. A study of hand sections discloses that in auxin-treated segments the intercellular spaces of the endocarp are filled with air (a normal situation), while in the control segments the spaces are filled with liquid. This suggests that the increased longevity of bean segments in vitro results from an effect of auxin on maintenance of membrane properties. In the absence of added auxin movement of solutes and water into the intercellular spaces results. This water-logging of the spaces is followed by dissolution of the middle lamella (cellular dissociation).

Permeability changes occur only in the endocarp (fig 1) of the bean segments, not in the exocarp. The different responses of adjacent tissues suggest that permeability changes could be involved in abscission phenomena. Experiments with Coleus show that 2 to 3 days after deblading, water-logging of the intercellular spaces and cellular dissociation occur in the abscission zone, followed by abscission. IAA applied distally to debladed petioles prevents these phenomena, supporting the hypothesis that auxin inhibits abscission by maintaining normal membrane properties.

This paper reports phenomena in fruit and leaf tissues in vitro which contribute to an understanding of the role of auxin in delaying tissue senescence.

[1] Received July 29, 1958.

[2] This work was supported by a Research Grant (RG 5273) from the Public Health Service, and was conducted in the Division of Biology, California Institute of Technology, Pasadena, California.

## METHODS AND EXPERIMENTAL RESULTS

BEAN SEEDS AS A SOURCE OF AUXIN: It has been reported that IAA occurs in young bean seeds (19). The following experiments were performed to ascertain if seeds function as a source of auxin in the maturing bean fruit. Bean pericarp segments were prepared with the seed removed and with the seed attached. The segments were kept on distilled water-moistened filter paper in Petri dishes at 25° C in the dark. Water-logging of intercellular spaces in the endocarp occurs in 2.5 to 3 days in deseeded segments, but not until 5 days in segments with the seed attached. The effect of the seed in these studies is comparable to the effect of IAA at a concentration of 4 ppm. The resemblance between the effect of the attached seed and of applied auxin in preservation of membrane properties suggests that in the bean fruit the seeds are a source of auxin, which acts to retard the occurrence of permeability changes. Observations on whole beans during storage show that the endocarp undergoes permeability changes and cellular dissociation, occurring 1st in the tissue between the seeds, and with continued aging progressing to the region where the seed is attached.

EXUDATION IN ENDOCARP TISSUE: It is consistently observed that conspicuous droplets of liquid appear on the upper and lateral surfaces of water-treated segments (fig 5). This is manifest after 4 to 5 days, or 1 to 2 days after water-logging occurs in the endocarp. This phenomenon does not occur in auxin-treated segments. These observations suggest the following explanation. The alteration in membrane properties in water-treated segments enables the leakage of solutes into the cell wall and intercellular space regions, which is accompanied by outward diffusion of water. When the intercellular region becomes filled with liquid, the continued movement of water into the intercellular region results in displacement of some of the intercellular contents as a surface exudate [3]. Since cellular dissociation is evident during the period when exudation occurs, the

[3] A subsequent publication will report on analyses of the exudate and pectin changes.

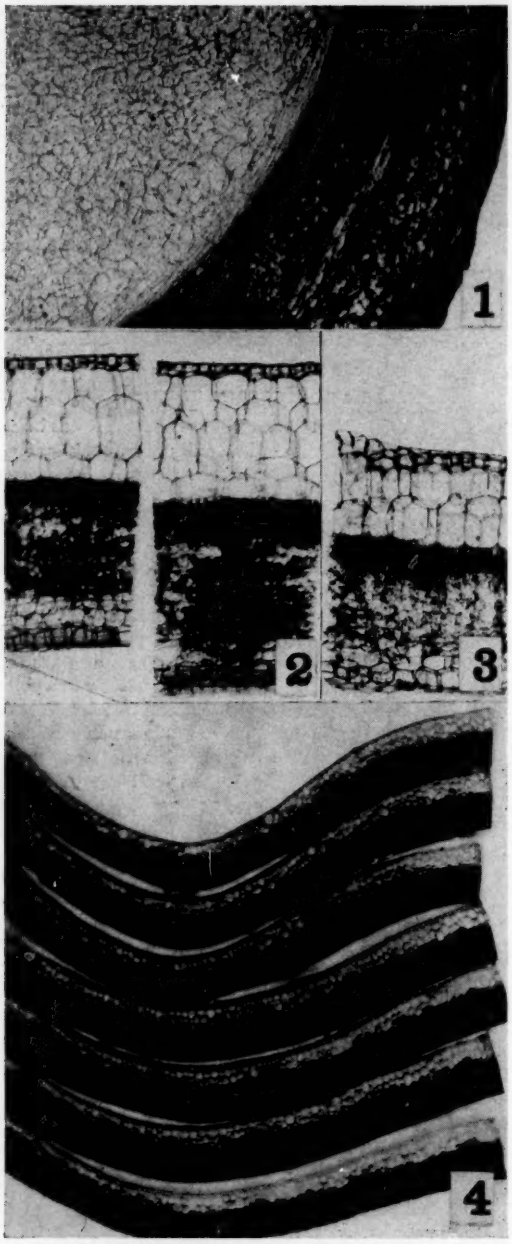


FIG. 1. Hand section of bean segment after 3 days of water-treatment. The intercellular spaces in only the endocarp are water-logged. The exocarp shows air-filled intercellular spaces (they appear black, owing to reflection phenomena). × 50.

FIG. 2. Hand section of a Rhoeo leaf slice after 28 days of auxin-treatment to illustrate the abundance of intercellular air in the spongy chlorenchyma. Anthocyanins show in the abaxial epidermis (bottom). × 50.

FIG. 3. Hand section of a Rhoeo leaf slice after 5 days of water-treatment. Intercellular spaces water-logged; anthocyanins have been lost by exosmosis. × 50.

FIG. 4. 1 mm thick Rhoeo leaf slices after 28 days of auxin-treatment. The slices show no evidence of permeability changes as evidenced by their turgid, healthy appearance, absence of liquid-logging of air spaces, and retention of anthocyanin pigments. × 10.

degradation of pectin in the middle lamella may be a factor contributing to the osmotic pressure of the intercellular medium.

PLASMOLYTIC STUDIES: The ability of cells to undergo plasmolysis and deplasmolysis has often been used as a criterion of selective permeability of cell membranes (6). In the following experiments the osmotic behavior of protoplasts of cells in which changes in permeability had occurred (water-treated endocarp) is compared with cells of fresh endocarp and auxin-treated endocarp in which there is no apparent indication of alteration in membrane properties.

Plasmolytic studies were made of hand sections of the endocarp of fresh bean pods, of endocarp after 3 to 7 days of water-treatment, and of endocarp after 3 to 7 days of auxin-treatment. The plasmolytic agent used was 0.75 M sucrose; 0.1 M sucrose was used for deplasmolysis. Under each of the 3 conditions plasmolysis and deplasmolysis is observed through 2 cycles. Despite the evidence for alteration in membrane properties in water-treated endocarp, the protoplasts retain characteristics of differential permeability. It became obvious, however, that plasmolysis occurs much more rapidly in water-treated than in auxin-treated tissue. The following procedure was used to determine the rate at which plasmolysis occurs in fresh, auxin- and water-treated endocarp.

The time for plasmolysis was measured from the time that 0.75 M sucrose was added at the edge of the coverslip, and drawn through using filter paper, until the protoplast had completely withdrawn from the cell wall. Deplasmolysis was recorded from the time of adding 0.1 M sucrose until the protoplast resumed contact with the cell wall. Observations on the rate of plasmolysis were made on cells at the edges of the sections. This procedure minimizes the effect of variation in thickness of hand sections, which have to be a few cell layers in thickness to ensure cells uninjured by sectioning.

The more rapid rate of plasmolysis of water-treated than of fresh or auxin-treated endocarp is illustrated in table I. The plasmolytic studies were repeated on numerous batches of beans over a period of 12 months. The lack of uniform response in auxin-treated segments among different experiments is probably attributable largely to batch-wise variation in beans and their ability to respond to auxin. Regardless of the variability, it is clear that the time required for

TABLE I

RATES OF PLASMOLYSIS IN FRESH, WATER- AND AUXIN-TREATED ENDOCARP

| TISSUE | TIME FOR PLASMOLYSIS* | TIME FOR DEPLASMOLYSIS |
|---|---|---|
| Fresh endocarp | 15–25 minutes | 10–15 minutes |
| Water-treated endocarp (3–7 days) | 15 seconds to 3 minutes | 1–3 minutes |
| Auxin-treated endocarp (3–7 days) | 4–20 minutes | 4–15 minutes |

* Ranges of observations on rate of plasmolysis based on 8 samples of fresh tissue; 18 of water-treated tissue; 15 of NAA-treated tissue.

plasmolysis of water-treated tissue is significantly less than for fresh or auxin-treated tissue. The increased rate of plasmolysis in water-treated tissue provides further evidence of a change in permeability.

An interesting observation associated with addition of 0.75 M sucrose to sections of water-treated endocarp is the instantaneous and marked increase in refractivity of the protoplast, followed by plasmolysis in from 15 seconds to 3 minutes. The rapid increase in refractivity of the protoplast may be related to a greater permeability of the plasma membrane than of the tonoplast. MacRobbie and Dainty (15) report that in Nitellopsis flux through the plasma membrane is 20 to 100 times that through the tonoplast. It seems that the increased refractivity depends upon a change in cytoplasmic state, for it precedes the occurrence of visible contraction of the protoplast (plasmolysis). Whether the rapid loss of water from the cytoplasm or rapid entry of the plasmoliticum into the cytoplasm is the cause of the change in refractive index of the cytoplasm has not been determined. In fresh tissue, increase in refractivity takes ca. 20 minutes, attending retraction of the protoplast from the wall.

The effect of cellular dissociation on severing cytoplasmic connections between cells does not appear to have a significant effect on the rate of plasmolysis, as plasmolysis of dissociated cells is initially crenulate (as in fresh tissue), suggesting attachment of the protoplast to the cell wall. Plasmolytic studies of endocarp cells after 3 days and after 4 to 7 days of water-treatment reveal no consistent difference in the time required for plasmolysis, even though cellular dissociation is very conspicuous during the latter period. These observations suggest that the difference in magnitude between rates of plasmolysis in water- and auxin-treated segments is not appreciably affected by cellular dissociation in the former.

The occurrence of water-logging of air spaces, conspicuous exudation, and the increase in rate of plasmolysis in water-treated bean endocarp provide clear evidence of an alteration in membrane permeability, a process which is delayed by an exogenous source of auxin. It is apparent, however, from the response to a plasmolytic medium that the membranes still exhibit differential permeability. It may be inferred that the ability of cells to undergo plasmolysis and deplasmolysis may not give a full indication of properties of cell membranes unless the rate factor is considered.

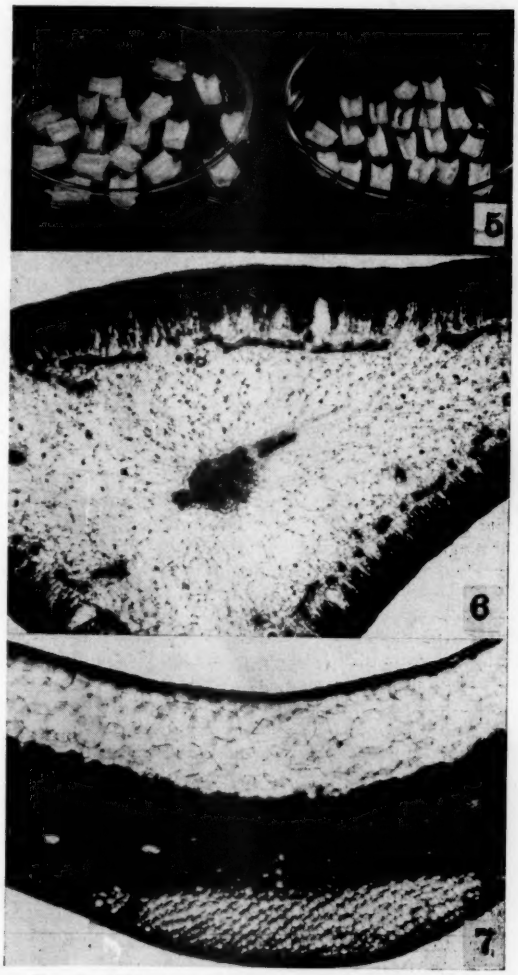


FIG. 5. Dish on left shows bean endocarp segments after 5 days of water-treatment. Note the droplets of liquid exudate on surface of segments. Dish on right shows segments after 5 days of auxin-treatment, with no exudation. × 3/8.

FIG. 6. Hand section of a leaf of Mesembryanthemum. Description in text. × 20.

FIG. 7. Hand section of Rhoeo leaf (midrib region) depicting the colorless, compact adaxial tissue, central spongy chlorenchyma (appearing black due to extensive air-filled intercellular spaces), and the adaxial tissue zone containing anthocyanins. × 40.

RESPIRATORY STUDIES: When bean segments are prepared without asepsis the water-treated segments succumb to bacterial contamination after 5 days, or 2 days after the completion of water-logging. In contrast, auxin-treated segments do not become contaminated until 12 to 20 days elapse (22), again following the ultimate change in membrane properties. It seems that contamination results from the favorable substratum provided by the leakage of cellular substances. Experiments performed with asepsis reveal that cells of water-treated endocarp appear normal cytologically over a 10-day period, notwithstanding the dissociation of the endocarp into a loose mass of cells. Thus it seemed attractive to conduct respiratory studies on fresh and on water- and auxin-treated endocarp to ascertain the effect of alterations in permeability on rate of respiration.

Bean segments were prepared aseptically with exocarp and mesocarp removed and kept in the dark at 25° C in Petri dishes on filter paper moistened with distilled water or a 50 ppm solution of NAA. The removal of the outer tissues provided segments of homogeneous endocarp for respiratory studies. Segments were removed at 1-day intervals, quartered, blotted, weighed and placed into Warburg vessels. Samples were run in triplicate, each vessel containing 300 to 600 mg of tissue.

It can be seen from a comparison of the curves in figure 8 that except for the 1st 3 days the $Q\,O_2$ for the auxin- and water-treated endocarp is similar throughout the experiments (18). There occurs a gradual decline in the rate of respiration until the 6th day at which time the rate levels off. Similar results were obtained in further experiments. RQ determinations yield like results for both treatments, ranging from ca. 1.11 at the beginning of the experiment to 1.06 on the 6th day. There is no effect on oxygen uptake of sucrose (0.04 M), glucose (0.02 M) or 0.02 M phosphate buffer at pH 7, or combinations thereof, whether the materials are exposed to the added substrates for a 2-hour period or overnight. In both water- and auxin-treated endocarp, however, there is an increase in the RQ (1.06 to 1.12) when sucrose is added (2). These results demonstrate that endocarp in which permeability changes and cellular dissociation had occurred may exhibit a rate of respiration equal to that of tissue of similar age treated with auxin to maintain membrane properties. As the following experiments show, however, water-treated segments undergo complete loss of protoplasmic organization and respiratory activity over longer periods of time, becoming disorganized at least 20 days prior to auxin-treated segments.

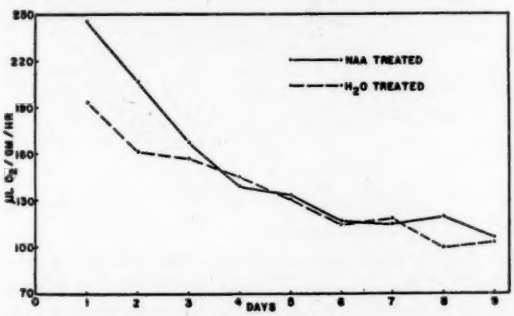


FIG. 8. Endogenous respiration of bean endocarp segments.

Auxin- and water-treated endocarp segments prepared as previously described were stored in the dark at 25° C for 5 days and then at 5° C in the dark for 20 days. At the end of this period respiratory studies were made 2 hours after removal of the materials from cold storage. The water-treated segments show no oxygen uptake. Further, a study of hand sections reveals protoplasmic disorganization. In contrast, auxin-treated segments show an uptake of 80 $\mu$l $O_2$/g/hr, a rate comparable to the level exhibited by segments during days 7 through 9 (fig 8). A similar $Q\,O_2$ (133 $\mu$l $O_2$/g/hr) is shown by auxin-treated segments after more prolonged storage, namely, for 5 days at 25° C and 40 days at 5° C. No respiratory measurements were made on auxin-treated segments beyond 45 days, but their appearance up to 60 days suggested that they had biological activity.

The conditions of the above experiment provide a means of analyzing the effect of permeability changes on longevity of bean segments. The initial storage period at 25° C provides adequate time for the occurrence of permeability changes and cellular dissociation in water-treated segments. The subsequent cold period a) reduces the possibility of contamination and b) lowers metabolic activity so that any effect of permeability changes on leakage of cellular materials is evident before the depletion of metabolites limits biological organization. The auxin-treated segments exhibit respiratory activity 20+ days longer than do water-treated segments. It seems plausible to regard permeability changes in water-treated segments as permitting excessive leakage of cellular materials, which could bring about deficiencies of metabolites sufficient to cause cessation of biological activity. As a partial test of this hypothesis the following dry weight determinations of the diffusate from water- and auxin-treated segments were made. The term diffusate refers to solutes which leak from the segments into the external medium.

DIFFUSATE STUDIES: Bean endocarp segments were prepared aseptically and stored as described above (i.e., in the dark at 25° C for 5 days; thereafter in the dark at 5° C). In view of variations in the initial size and water content of trimmed segments, the most reliable estimate of the relative amount of leakage may be obtained from comparison of the ratio of the final dry weights of diffusate to segments for each of the batches. To this end, 14 segments of water- and auxin-treated tissue respectively were removed at 7, 14 and 21 days for determination of dry weights of segments and diffusates. The diffusates were washed from the Petri dishes with distilled water, filtered through Whatman no. 1 filter paper, and diffusates and segments were dried in vacuum over

sulfuric acid. The diffusates from auxin-treated segments are clear, in contrast with the turbid-white character of diffusates from 14- and 21-day-old water-treated segments. The final dry weights of segments and diffusates are shown in table II.

As is shown in table II there is, over the 21-day period, ca. 3 times more leakage from water-treated segments than from auxin-treated segments. These data are consistent with the hypothesis that the earlier loss of respiratory activity in water-treated segments results from excessive leakage, and that added auxin, through its effect in maintenance of membrane properties, delays this process.

PERMEABILITY STUDIES ON LEAF TISSUES IN VITRO: The evidence that auxin affects membrane permeability in bean endocarp tissue and in the abscission zone in Coleus, prompted investigation of the effect of auxin on leaf tissues. The leaves of *Mesembryanthemum sp.* and *Rhoeo discolor* were used because their thickness makes them adaptable to the study of fresh sections. It should be added that Rhoeo was also favored because of the anthocyanin pigments it contains.

*Mesembryanthemum*: Leaves were sterilized in 10 % Clorox for 10 minutes and then cut transversely into segments 5 to 6 mm in length. The segments were transferred aseptically into Petri dishes on filter paper moistened with sterile distilled water or a solution of 4 ppm NAA, and stored in the dark at 25° C. The appearance of a fresh transection of the leaf is illustrated in figure 6. The central zone is occupied by colorless mesophyll tissue having fairly prominent intercellular spaces. External to this is a layer of palisade chlorenchyma, limited outwardly by the epidermis.

In water-treated segments water-logging of intercellular spaces in the central zone occurs in 4 to 5 days. Within 24 hours after water-logging the rigidity of this tissue is lost, and browning is apparent. In contrast, treatment of the leaf segments with 4 ppm NAA delays water-logging of the air spaces and browning until 9 days. This effect of auxin in maintenance of membrane properties is best shown in segments of young leaves.

*Rhoeo*: Leaves were sterilized in 10 % Clorox for 10 minutes, after which a longitudinal segment ca. 12 mm in width containing the midrib was removed and cut transversely into slices 1 mm thick. The slices were washed in sterile distilled water to remove brei released from cells injured during sectioning. They were then placed aseptically into Petri dishes containing distilled water or a 4 ppm NAA solution and kept in the dark at 25° C.

Figure 7 illustrates a section of a Rhoeo leaf. The leaf consists of 3 horizontally arranged tissue zones. In the region of the adaxial surface there is a compact colorless tissue, 2 to 5 cell layers in depth, lacking intercellular spaces. The abaxial region consists of a compact tissue varying from 1 to several cells in thickness, containing anthocyanin pigments. The central zone is occupied by photosynthetic tissue with loose cellular arrangement and large intercellular spaces.

Within 6 days there are several obvious degenerative changes in water-treated Rhoeo slices. After 4 days browning is evident in the colorless adaxial tissue region, and the intercellular spaces in the photosynthetic tissue become water-logged (fig 3). Cellular dissociation follows rapidly in the photosynthetic tissue. By the 6th day the anthocyanins leak into the medium. The identity of the pigments was demonstrated by chromatographic analysis. The diffusate was treated with 0.1 % HCl, concentrated by distillation in vacuum and chromatographed on Whatman no. 1 paper in a butanol : acetic acid : water (4:1:5) solvent, along with a similarly concentrated control consisting of pigments washed from fresh leaf slices with 0.1 % HCl. The $R_f$ values were the same in all cases.

In contrast, auxin-treated leaf slices show no browning, water-logging of the air spaces or loss of anthocyanins for a period of 30 to 35 days. Figure 2 depicts hand sections of Rhoeo leaf slices after 28 days of auxin treatment. Figure 4 illustrates a group of 1 mm thick leaf slices as they appear after being maintained on a solution of 4 ppm NAA for 28 days.

As in bean endocarp it seems clear that the addition of auxin enables maintenance of normal membrane properties in Mesembryanthemum leaf segments and Rhoeo leaf slices. The 3 different changes, which occur in Rhoeo in the absence of added auxin, may be attributed to permeability changes: a) The water-logging of the prominent air spaces in the photosynthetic tissue, followed by cellular dissociation, seems

TABLE II

ANALYSIS OF DIFFUSATE FROM NAA- AND WATER-TREATED ENDOCARP*

| TREATMENT | 7 DAY | | | 14 DAY | | | 21 DAY | | |
|---|---|---|---|---|---|---|---|---|---|
| | DRY WT SEGM | DRY WT DIFFUSATE | PERCENTAGE** DRY WT | DRY WT SEGM | DRY WT DIFFUSATE | PERCENTAGE** DRY WT | DRY WT SEGM | DRY WT DIFFUSATE | PERCENTAGE** DRY WT |
| NAA | 0.1271 | 0.0037 | 2.9 % | 0.1150 | 0.0083 | 7.2 % | 0.1272 | 0.0154 | 12.1 % |
| $H_2O$ | 0.1100 | 0.0095 | 8.6 % | 0.1039 | 0.0187 | 18.0 % | 0.0875 | 0.0312 | 35.6 % |

* The figures are for batches of 14 bean segments, all weights in grams.

** Expressed as ratio of dry weight of diffusate to dry weight of segments.

to parallel a similar effect observed in bean endocarp and in the abscission zone of Coleus. b) The leakage of anthocyanins resembles the loss of such pigments from beet tissue when its membranes are injured by heat or other agents (8). c) The browning reaction may be attributable to permeability changes through an effect on enzyme-substrate relationships, e.g., polyphenols and polyphenolases (21).

It is of interest to note that the tissue containing anthocyanins and the colorless tissue, both of which are compact and devoid of intercellular spaces, do not exhibit cellular dissociation. Yet in each there occurs a phenomenon suggestive of changes in membrane permeability.

## Discussion

Experiments with segments of bean endocarp, and with leaf slices of Rhoeo and Mesembryanthemum, provide clear evidence that auxin, long regarded as a growth hormone, also effects the preservation of certain membrane properties. To what extent auxin may effect maintenance of membrane properties in root and stem tissues is being investigated. The auxin-effects described in this paper are manifested under conditions where little or no growth occurs.

The evidences of changes in permeability in the absence of added auxin derived from studies on bean endocarp and certain leaf tissues are several, as follows: 1) water-logging of intercellular spaces within a few days, 2) microbial contamination of tissues in absence of asepsis, 3) a considerable decrease in the time required for plasmolysis of bean endocarp, 4) conspicuous exudation at the surface of endocarp segments, 5) the loss of anthocyanins from Rhoeo leaf slices, 6) the occurrence of browning in Rhoeo and Mesembryanthemum leaf tissues and 7) the leakage of ca. 36 % of the final dry weight in bean endocarp attended by a cessation of respiration. In the experimental materials these phenomena are inhibited by NAA for up to 30 days. Thus it is attractive to consider that auxin may act as a factor retarding senescence in leaf and fruit tissues in vivo, by effecting preservation of membrane properties. Recent studies (20) demonstrate that kinetin increases longevity of detached Xanthium leaves by deterring proteolysis. The present investigation shows that kinetin has no effect on the longevity of bean segments.

The water-logging of intercellular spaces is best explained by considering that a change in membrane permeability occurs, thereby permitting a movement of certain solutes, and hence of water into the intercellular spaces (to satisfy the diffusion pressure deficit). It might be argued that water from the medium injects the air spaces in bean endocarp. This, however, is negated by the fact that this phenomenon occurs in the abscission zone in Coleus (and in Fuschia and Cestrum, unpublished) where the tissue is not exposed to an external source of water.

The water-logging of intercellular spaces and increased rate of plasmolysis in water-treated endocarp are evident preceding cellular dissociation. These observations argue against an interpretation that water-logging is an effect of hydrolysis of pectins in the middle lamella resulting in the formation of a hydrated pectin jelly in the air spaces. The fact that the $Q\,O_2$ of water-logged tissue parallels that of auxin-treated tissue for 9 days suggests no drastic disorganization of the protoplasts, despite alterations in permeability. Further, during the same interval, protoplasmic organization in water-logged tissue appears normal. Upon prolonged aging (20 days), however, the permeability changes accelerate protoplasmic disorganization, as evidenced by the loss of respiratory activity in water-treated segments, attended by marked leakage. Auxin effectively obviates disorganization for over 45 days. Thus it is concluded that auxin delays senescence by maintaining membranes in their normal state.

The conspicuous browning in the colorless adaxial tissue in Rhoeo leaf slices attends leakage of anthocyanins from the abaxial tissue zone and water-logging of the intercellular spaces in the centrally situated chlorenchyma. Thus it is interpreted as an effect of alterations in membrane properties. A browning effect analogous with this is the so-called "tea fermentation," attributed to cytoplasmic disorganization which enables vacuolar polyphenols to come into contact with cytoplasmic polyphenolases (21). Browning has been reported in maturing apples (10) and attributed to "greater access of polyphenols to enzymes." Also, it may be mentioned that the bruising of apples without rupture of the skin brings on browning, again an analogy with tea fermentation.

These reports together with the present observations suggest that browning in aged or injured fruit and leaf tissues may be an effect of permeability changes. An additional consequence of unnatural polyphenolase action in vivo may be the inhibition of oxidative phosphorylation owing to the accumulation of oxidized polyphenols (13), thus accelerating senescence.

The demonstration of auxin-permeability relationships in bean and leaf tissues prompted studies of certain drupes and berries to see if maturation phenomena such as cellular dissociation, increase in soluble pectins, the climacteric rise in respiration (1), and increased malate decarboxylation (9, 17) are preceded by permeability changes. Although this investigation is still in progress, preliminary observations support this view as evidenced by the occurrence of water-logging of air spaces in tissue segments in vitro as well as in intact maturing fruits. Thus, the question is raised as to whether such phenomena are effects of permeability changes. It is of interest to point out that Blackman (1) and Kidd and West (11) suggested that the climacteric may be related to permeability changes. Further investigation is needed on the role which permeability changes may play in ripening fruits. The demonstrated water-logging of air spaces in maturing fruit tissues, however, makes it attractive to consider changes in membrane permeability as being the cause of phenomena associated with the maturation of certain fruits.

Several investigators have shown that divalent ions reduce leakage of cellular materials (12, 14). Laties (12) reports that exposure of tissue to a monovalent salt solution results in water-injection and loss of all aspects of semipermeability. These effects are prevented if $10^{-4}$ M $CaSO_4$ is in the bathing medium. The storage of bean endocarp in $5 \times 10^{-4}$ $CaSO_4$, however, has no effect compared to water, nor does it prolong maintenance of membrane properties when added to an auxin solution. According to Helder (8) segments of plant tissue suspended in distilled water undergo leakage of both organic and inorganic substances. The storage of bean endocarp in 0.1 to 0.3 M sucrose or mannitol does not prevent permeability changes. Thus, it is concluded that the auxin-membrane permeability relationship differs from the effect of monovalent ions or distilled water on permeability.

The fact that water-treated endocarp plasmolyzes more rapidly than fresh or auxin-treated endocarp does not establish that the permeability changes occur in both vacuolar and plasma membranes. Since it has been shown that the plasma membrane is 100 times more permeable than the tonoplast (15), the increased rate of plasmolysis could be accounted for by a change in permeability of the tonoplast only. The instantaneous increase in cytoplasmic refractivity of water-treated endocarp when exposed to the plasmoliticum, however, can best be explained by a marked change in permeability of the plasma membrane, inasmuch as the increase in refractivity precedes plasmolysis. Considering the apparent ubiquity of membrane structure the possibility should be considered that mitochondrial, as well as plasma and vacuolar, membranes are affected.

Although a scheme had been proposed earlier (24) by which auxin (IAA) could affect membrane permeability directly, the possibility must also be considered that auxin effects maintenance of membrane properties through some metabolic pathway. An interaction between auxin and iodoacetate affecting membrane permeability is shown by Christiansen (5). He reports that auxin-stimulated growth of pea stem segments is blocked by iodoacetate, and is attended by a marked leakage of cellular substances. In the presence of iodoacetate or auxin alone there is no leakage. That this may differ from the permeability changes described in this paper is indicated by its reversibility when iodoacetate is removed. It is not known if there was an accompanying water-logging of the intercellular spaces.

Recent work by Galston on IAA oxidase gradients in shoots (7) suggests that the occurrence of permeability changes in bean endocarp, and in abscission zone tissue in Coleus, may be related to a higher rate of IAA destruction than in contiguous tissues. Current work reveals that IAA oxidase activity in the brei of endocarp is 3 to 4 times higher than in the contiguous exocarp. IAA oxidase determinations to date on abscission zone and adjacent tissues have been inconclusive.

## Summary

Studies of fruit and leaf tissues in vitro demonstrate that auxin acts to delay senescence. The experimental work shows the dependence of these tissues on auxin for maintenance of membrane properties. Whereas in water-treated tissues permeability changes occur in ca. 5 days, the addition of auxin delays this process for up to 30 days. In this paper evidences of alterations in membrane permeability in the endocarp in the absence of added auxin are 1) the rapid rate of plasmolysis in water-treated as compared with fresh and auxin-treated endocarp segments, 2) the conspicuous exudation which appears on the surface of water-treated endocarp segments, and 3) the considerably greater leakage of cellular contents from water-treated than from auxin-treated segments.

Respiratory studies of endocarp segments reveal no significant difference under the 2 treatments over a 9-day period. Prolonged studies show that water-treated endocarp loses respiratory activity by 22 days, while auxin-treated endocarp exhibits a moderate $Q\,O_2$ for 45 days or more. The earlier loss of activity in the former is believed attributable to the effect of permeability changes on excessive leakage of cellular contents.

Striking effects are achieved in delaying senescence of 1 mm thick Rhoeo leaf slices with 4 ppm NAA. Water-treated slices exhibit degenerative phenomena within 5 days, such as liquid-logging of intercellular spaces, cellular dissociation, browning, and loss of anthocyanins. These sequences are delayed for up to 30 days by the addition of auxin only. Similar effects are obtained with segments of leaves of Mesembryanthemum. The possible relationship between permeability changes and phenomena attending senescence of certain fleshy fruits is discussed.

The author wishes to gratefully acknowledge the helpful discussions with Dr. G. Laties during the course of the investigation, and express appreciation to Drs. A. J. Haagen-Smit and G. Laties for reviewing the manuscript.

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# SOME METABOLIC RESPONSES OF ALFALFA SEEDLINGS TO FREEZING [1, 2]

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Species and varietal differences in cold hardiness or susceptibility have been studied for years and although many plant reactions to low temperatures have been reported, the physiological and biochemical bases for recovery from low temperature stresses are still not clear. The extensive literature on methods of testing for winter hardiness have been reviewed by Dexter (2), and Levitt (7, 8) has reviewed numerous papers pertaining to physiology of hardening and frost hardiness.

Hansen and Nossal (4) have shown that in yeast, freezing not only increases permeability but also markedly alters enzyme structure and cellular morphology. They showed that nucleotide coenzyme-enzyme bonds are destroyed by freezing, and they cite unpublished data indicating that in frozen cells oxidative phosphorylation is abolished. The latter has also been demonstrated in cell-free extracts of bacterial particles (1). With succinate as a substrate, oxygen uptake of the particles was unaffected, yielding a very low ratio of phosphorus esterified to oxygen consumed.

The hardened cell or tissue is better able to withstand cold injury than is the unhardened, as shown by many past studies. Whether or not the physiological processes required for renewed growth after freezing are common to tissue which has been hardened and to unhardened tissue is not fully known. Some effects of different hardening conditions on respiration and regrowth of alfalfa will be presented elsewhere (11). The present paper reports the results of investigations into the respiratory metabolism of alfalfa seedlings and mitochondria in relation to freezing.

## MATERIALS AND METHODS

Seeds of Teton, a hardy variety of alfalfa (*Medicago media* L.), were germinated in plastic boxes on germination blotters in a dark germinator at 20° C for 4 to 5 days, at which time the radicle was 2 to 3 cm long, and the cotyledons had not yet broken the seed coat. Seedlings of alfalfa were employed at this stage of development in order to obtain a maximum amount of metabolically-active tissue. Relatively

[1] Received September 3, 1958.
[2] Journal paper no. 406 of the South Dakota Agricultural Experiment Station.

large numbers of individual plants could be included in each experiment providing at least a statistical control over genetic variability. The ability to rapidly produce large amounts of tissue grown under constant conditions also favored the use of young seedlings.

When seedling metabolism was being studied, 12 to 15 seedlings were transferred from the germinator to tared Warburg vessels containing the appropriate buffer and additives. Fresh weights were obtained by reweighing the Warburg vessels, thus avoiding excess handling of the seedlings. In most experiments, the seedlings were used with the cotyledons and seed coat intact. Sodium penicillin–G was added routinely to the Warburg flasks (0.5 mg/ml) to reduce contamination by bacteria. Most of the experiments were of sufficiently short duration so that contamination was not a serious problem.

Freezing treatments were accomplished by placing the Warburg flasks containing the experimental seedlings into a freezing chest at −18° C. Duration of freezing was varied from 0 to 96 hours, with a 60-minute freeze employed in most experiments. The seedlings were frozen within 5 minutes after being placed in the freezer. They were thawed at room temperature in the Warburg flasks and equilibrated for 20 to 30 minutes at 30° C in the conventional Warburg apparatus.

Mitochondria were isolated from alfalfa seedlings according to a modification of the method of Price and Thimann (9) using an extraction medium 0.3 M in sucrose, 0.02 M in phosphate buffer at pH 7.1, and 0.001 M in ethylene diamine tetraacetate. The homogenate produced by macerating the seedlings with a mortar and pestle or a Waring blendor was separated into plastids ($P_w$), mitochondria ($M_w$), and supernatant ($S_2$) by differential centrifugation. Low speed (500 × G) was used to isolate the $P_w$ fraction and high speed (10,000 × G) for the $M_w$ fraction. Freezing treatments were conducted by freezing the seedlings, the homogenate, the isolated $M_w$, or $M_w$ plus $P_w$ or $S_2$ prior to isolation of $M_w$, for determination of the oxygen uptake.

Oxygen uptake of the seedlings and the isolated cell fractions was determined by the usual manometric methods. Carbon dioxide evolution was determined by the "direct method" of Warburg (12). Readings taken at 5-minute intervals for 1 hour or more were plotted against time, and the linear rate of oxygen uptake so determined was expressed as microliters of oxygen uptake per hour ($Q_{O_2}$). Respiration was constant over a 2-hour period. Respiratory activity was calculated both on a fresh weight (FW) and total nitrogen (N) basis. The semimicro Kjeldahl procedure of Willits and Ogg (13) was used to determine total nitrogen.

KCN-KOH mixtures were prepared according to Robbie (10) to produce the desired concentration in the reaction medium when placed in the center well and equilibrated with closed stopcocks for 1 hour at 30° C (5).

## Experimental Results

The effect of freezing upon respiration in Teton alfalfa seedlings was studied both in the intact seedlings and in the cell-free particulates of the seedlings. It was established early in these investigations that freezing and thawing was markedly injurious to the respiratory activity of the tissues employed. A water-soaked appearance was noted in frozen and thawed seedlings, and it was of interest to determine to what extent cold injury was reflected in the oxidative activity of the mitochondria.

Cell Fractions: Preliminary studies indicated that an optimum endogenous oxygen consumption by the mitochondria was obtained when the seedlings were disintegrated in 0.3 M sucrose, but when succinate was employed as a substrate, higher rates of respiratory activity were obtained with 0.6 M sucrose in the homogenizing medium (table I). The relatively high endogenous activity of alfalfa mitochondria is quantitatively similar to that obtained by Freebairn et al (3) for a variety of plant species.

Table I

Effect of Sucrose Concentration in the Extraction Medium on Oxygen Uptake by Teton Mitochondria *

| | $Qo_2$ (FW) ** | | $Qo_2$ (N) ** | |
|---|---|---|---|---|
| Sucrose | Endogenous | Succinate | Endogenous | Succinate |
| *M* | *µl/g/hr* | | *µl/mg/hr* | |
| 0.00 | 25.4 | 16.4 | 155.9 | 105.9 |
| 0.15 | 27.5 | 25.2 | 141.2 | 129.0 |
| 0.30 | 41.1 | 33.3 | 194.3 | 157.8 |
| 0.45 | 38.3 | 33.1 | 191.6 | 165.6 |
| 0.60 | 31.7 | 38.7 | 170.1 | 207.9 |

* Reaction medium consisted of 0.001 M AMP, 0.02 M, glucose, 0.006 M $MgCl_2$, 1 mg/ml hexokinase, $3.3 \times 10^{-4}$ M DPN, and 0.04 M sodium succinate.

** Oxygen uptake is expressed on the basis of fresh weight (FW) and total nitrogen (N).

Although oxygen uptake of isolated mitochondria varied from extremes of 115 to 200 microliters per mg N per hour from one experiment to another despite close control of growth and extraction conditions, agreement between replicates within each experiment was good. As shown in table II, respiratory activity of the plastid fraction ($P_w$) was appreciable, indicating that extraction of mitochondria may have been incomplete. Oxygen uptake by the supernatant ($S_2$) was measurable but small.

The data of table II illustrate the effect of direct freezing of the 3 major cell constituents upon their subsequent respiratory activity. Depression of oxygen uptake in the mitochondrial fraction was small, but was evident both in endogenous and succinate respiration. The decrease in oxygen uptake in the mitochondria was partially offset by small increases in the frozen plastid and supernatant fractions. Total

TABLE II

ENDOGENOUS AND CORRECTED SUCCINATE OXYGEN UPTAKE OF FROZEN AND UNFROZEN CELL CONSTITUENTS

| CELL FRACTION | $Qo_2$ (N) | | | |
|---|---|---|---|---|
| | ENDOGENOUS | | SUCCINATE | |
| | UNFROZEN | FROZEN | UNFROZEN | FROZEN |
| | *μl/mg/hr* | | *μl/mg/hr* | |
| $M_w$ | 163 | 131 | 142 | 109 |
| $P_w$ | 102 | 114 | 140 | 154 |
| $S_2$ | 36 | 49 | 0 | 0 |
| Total | 301 | 294 | 282 | 263 |

oxygen uptake in the frozen and unfrozen samples did not differ greatly.

There was no marked difference between hardy and non-hardy varieties with respect to the oxygen uptake of mitochondria isolated from frozen and unfrozen seedlings (table III). Arizona Chilean, a non-hardy variety, had lower mitochondrial activity than did the hardy Teton but there was no appreciable effect of freezing in either variety.

TABLE III

ENDOGENOUS ACTIVITY OF MITOCHONDRIA ISOLATED FROM FROZEN AND UNFROZEN TETON AND ARIZONA CHILEAN ALFALFA SEEDLINGS

| VARIETY | $Qo_2$ (N) | |
|---|---|---|
| | UNFROZEN | FROZEN |
| | *μl/mg/hr* | *μl/mg/hr* |
| Arizona Chilean | 80.2 | 81.5 |
| Teton | 115.6 | 111.3 |

It appears that freezing the mitochondria in the intact seedling or following their isolation has no great effect upon their subsequent oxidative ability in vitro, despite the fact that respiration of frozen intact seedlings was markedly depressed, as will be shown in the next section. It thus seemed possible that a water soluble respiration inhibitor could be formed in the seedlings by the freezing or thawing process which would be removed during the isolation of the mitochondria. To test this hypothesis, additions of $S_2$, $P_w$ and $S_2$ plus $P_w$ were made to the frozen and unfrozen mitochondria just prior to determination of oxygen uptake (table IV). These data show the slight effect of freezing in lowering the respiration of the mitochondria. Addition of $S_2$ or $S_2$ plus $P_w$ to either the frozen or unfrozen mitochondria produced marked stimulation, but addition of $P_w$ alone had no effect. Similar experiments were conducted in which frozen and thawed $P_w$ and $S_2$ were added to frozen and unfrozen mitochondria with results of the same nature as those shown in table IV.

TABLE IV

EFFECT OF FREEZING AND ADDITION OF OTHER CELL FRACTIONS ON THE ENDOGENOUS OXYGEN UPTAKE OF TETON MITOCHONDRIA

| TREATMENT | ADDITIONS | $Qo_2$ (N) |
|---|---|---|
| | | *μl/mg/hr* |
| Unfrozen | None | 202.0 |
| | $S_2$ | 376.0 |
| | $P_w$ | 191.0 |
| | $P_w + S_2$ | 386.0 |
| Frozen | None | 196.0 |
| | $S_2$ | 365.7 |
| | $P_w$ | 180.7 |
| | $P_w + S_2$ | 350.3 |

TABLE V

EFFECT OF FREEZING THE CELLULAR HOMOGENATE UPON OXYGEN UPTAKE OF THE HOMOGENATE AND ITS COMPONENT CELL FRACTIONS

| CELL FRACTION | TREATMENT | $Qo_2$ (N) |
|---|---|---|
| | | *μl/mg/hr* |
| Homogenate | Unfrozen | 225.6 |
| | Frozen | 188.1 |
| $M_w$ | Unfrozen | 160.8 |
| | Frozen | 122.3 |
| $P_w$ | Unfrozen | 37.2 |
| | Frozen | 37.9 |
| $S_2$ | Unfrozen | 60.4 |
| | Frozen | 69.3 |

The data presented in table V show the effect of freezing the homogenate of Teton seedlings upon its oxygen uptake and that of the cellular components isolated from parallel samples of unfrozen or frozen homogenates. A decrease in respiration of both the homogenate and $M_w$ and a slight increase in $S_2$ due to freezing were obtained. Part of the respiratory capacity lost from the homogenate and mitochondria was recovered in the $S_2$ fraction.

SEEDLINGS: Preliminary studies with Teton alfalfa seedlings showed a broad pH tolerance with an optimum respiratory activity at pH 5.4. All studies reported here were conducted at this pH. The effect of duration of freezing was studied to determine the maximum depression of respiration in frozen and thawed seedlings (table VI). Freezing of the seedlings was accomplished within 5 minutes at −18° C. Respiration was depressed slightly with 10 minutes of freezing, and a maximum was reached at 15 minutes. Continued freezing did not further lower oxygen uptake. Stimulation of respiration by dinitrophenol was evident in both frozen and unfrozen seedlings, and the degree of stimulation was not greatly affected by freezing. Oxygen uptake of frozen and thawed seedlings was 30 to 40 % that of unfrozen seedlings. Although respiration of mitochondria from frozen seedlings is only slightly reduced, as measured in these studies, the respiration of intact frozen seedlings is greatly depressed.

TABLE VI

EFFECT OF DURATION OF FREEZING AND DNP ON RESPIRATION OF TETON ALFALFA SEEDLINGS

| FREEZING TIME | $Qo_2$ (FW) 1ST HOUR (NO DNP) | $Qo_2$ (FW) 2ND HOUR (DNP ADDED) | INCREASE BY DNP |
|---|---|---|---|
| *minutes* | *μl/g/hr* | *μl/g/hr* | *%* |
| 0 | 295.6 | 366.2 | 23.9 |
| 10 | 221.1 | 265.3 | 20.0 |
| 15 | 109.2 | 132.4 | 21.2 |
| 20 | 100.7 | 117.1 | 16.3 |
| 30 | 122.9 | 154.1 | 25.4 |

The marked but incomplete depression of oxygen uptake by freezing in whole Teton seedlings suggested a possible disruption or blockage of the electron transport system. Cyanide was used to block cytochrome oxidase activity, and it was determined that maximum inhibition in unfrozen seedlings occurred at a cyanide concentration of $10^{-4}$ M. Table VII shows the effect of cyanide on oxygen uptake of frozen and unfrozen seedlings. Reduction of oxygen uptake was strong and quantitatively almost equal by both freezing and cyanide. When cyanide was applied to frozen tissue, no further appreciable reduction in respiration was obtained. These data indicate the possibility that freezing acts upon the cyanide-sensitive system in alfalfa seedlings. Further study will be required to evaluate this possibility.

TABLE VII

CYANIDE SENSITIVITY OF FROZEN AND UNFROZEN 4-DAY-OLD ALFALFA SEEDLINGS

| TREATMENT | CYANIDE CONCENTRATION | $Qo_2$ (FW) |
|---|---|---|
| | *M* | *μl/g/hr* |
| Unfrozen | 0 | 423.3 |
| Unfrozen | $10^{-4}$ | 175.8 |
| Frozen | 0 | 163.1 |
| Frozen | $10^{-4}$ | 144.7 |

It was further noted (table VIII) that susceptibility to freezing and cyanide exhibited similar trends. The older the seedlings, the more resistance was shown to both cyanide and freezing. The degree of depression of respiration was approximately equal for the 2 inhibitors.

Attempts to obtain any measurable sensitivity to diethyldithiocarbamate in either frozen or unfrozen seedlings were unsuccessful.

## DISCUSSION

The loss of respiratory activity consistently obtained with frozen seedlings was not in evidence when the sub-cellular particles considered to be the chief site of respiratory activity in the cell were studied. The lack of effect of freezing on oxygen uptake by mitochondria has been reported (1), but the reason for the differential response between the tissue and sub-cellular unit is not clear. Because quantitative isolation of mitochondria from alfalfa seedlings was not achieved, a balance sheet of respiratory activity cannot be presented.

The possibility of production of a respiratory inhibitor upon freezing and thawing cannot yet be excluded because the dilution involved in extraction of the mitochondria could conceivably reduce the concentration of a reversible inhibitor below its threshold value. In addition, loss of essential cofactors and metabolites through cell membranes injured by freezing (4) may contribute to the reduced respiration in seedlings.

It could be merely fortuitous that treatments by freezing or with cyanide produced equivalent reduction in rate of oxygen uptake. Nevertheless that result was consistent with the hypothesis that cyanide and freezing act upon the same system. Because no diethyldithiocarbamate sensitivity was detected, it is assumed that the frost-insensitive respiration occurs through some terminal oxidation pathway other than cytochrome or ascorbic acid. Further investigation is required to determine the precise pathways of respiration insensitive to freezing.

## SUMMARY

Investigations into the sensitivity of respiration of Teton alfalfa seedlings and their cell-free mitochondria are reported. It was found that although respiration of frozen and thawed seedlings was depressed 60 to 70 %, the oxygen uptake of mitochondria from frozen seedlings was only slightly reduced.

TABLE VIII

AGE OF SEEDLINGS IN RELATION TO SUPPRESSION OF RESPIRATION BY CYANIDE AND FREEZING

| AGE OF SEEDLINGS | $Qo_2$ (FW) UNFROZEN UNTREATED | $Qo_2$ (FW) UNFROZEN CYANIDE* | $Qo_2$ (FW) FROZEN UNTREATED | $Qo_2$ (FW) FROZEN CYANIDE * | DEPRESSION OF OXYGEN UPTAKE BY FREEZING | DEPRESSION OF OXYGEN UPTAKE BY CYANIDE UNFROZEN | DEPRESSION OF OXYGEN UPTAKE BY CYANIDE FROZEN |
|---|---|---|---|---|---|---|---|
| *hours* | *μl/g/hr* | *μl/g/hr* | *μl/g/hr* | *μl/g/hr* | *%* | *%* | *%* |
| 48 | 742.2 | 160.4 | 169.1 | 106.2 | 77.2 | 78.5 | 37.2 |
| 72 | 423.1 | 122.7 | 80.0 | 70.8 | 81.1 | 71.0 | 11.5 |
| 96 | 326.8 | 79.6 | 77.1 | 73.5 | 77.1 | 75.6 | 2.0 |
| 120 | 284.6 | 87.5 | 80.2 | 65.8 | 71.8 | 69.3 | 18.0 |

* $10^{-4}$ M cyanide.

Equivalent inhibition of oxygen uptake was obtained with cyanide and freezing, but no additional depression of respiration was found when cyanide was added to frozen seedlings. It was suggested that freezing abolishes the cyanide-sensitive respiration.

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# METABOLISM OF $C^{14}$-BICARBONATE, $P^{32}$-PHOSPHATE, OR $S^{35}$-SULFATE BY LETTUCE SEED DURING GERMINATION [1]

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The time course of germination of lettuce seed has been divided into the following phases: imbibition, activation, mitosis, protrusion of radicle, and growth (5). Although some enzymes have been found in extracts of seeds throughout the course of germination, very little is known of the metabolism of intact seeds during the phases before protrusion. It has been shown that lettuce seeds respire in the earliest phases of germination (5, 9, 13). Presumably, germination-promoting light and chemicals act during the early phases preceding protrusion. We used radioactive bicarbonate, phosphate, and sulfate in the experiments reported here to explore the metabolism of lettuce seeds during the phases of germination preceding radicle protrusion.

## Materials and Methods

Seeds of *Lactuca sativa*, var. Grand Rapids, were germinated in covered 5.5-cm Petri dishes, each containing a pad of filter paper moistened with 1 ml of solution containing 100 $\mu$c of $NaHC^{14}O_3$ buffered at pH 9 with 0.05 M Tris (hydroxymethyl)aminomethane 0.005 M in $Cl^-$, or 60 $\mu$c of $NaH_2P^{32}O_4$ in distilled water at pH 5.8, or 100 $\mu$c $Na_2S^{35}O_4$ in distilled water at pH 5.8. Radioisotopes were purchased from the Oak Ridge National Laboratory. Specific activities of the tracers at the beginning of the experiments were approximately 160 to 1200 mc of $C^{14}$ per gram of carbon and 10,000 to 40,000 mc of $P^{32}$ per gram of phosphorus. The $S^{35}$ was carrier free. All experiments were performed at 21 to 22° C. Light treatments (room lighting or far-red) were given continuously. Unless otherwise designated, seeds were germinated under 20 ft-c of white light. Seeds designated as punctured were pricked through the surface of the seed coats covering the cotyledons with a number 8 needle so that the embryo was not touched and viability was unaffected.

Six to ten seeds were extracted by boiling for several minutes in 3 ml of 85 % ethanol and then in 3 ml of water. Unless otherwise indicated, the alcohol- and water-soluble extracts were combined for analysis by two-dimensional paper chromatograhy as described by Bassham and Calvin (1). When indicated, the following solvents were also used for developing one-dimensional chromatograms: methanol : 80 % formic acid : water (80 : 15 : 5 v/v); *tert*-amyl alcohol saturated with water; ethanol : glacial acetic acid (95 : 5 v/v) (3).

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## Results

Under the conditions of room temperature and lighting, the seeds used in these experiments entered the protrusion phase around 15 hours after the beginning of imbibition, whether or not they had been punctured. Imbibition of water, as determined by weighing groups of 100 seeds, was complete after the 1st 4 hours in unpunctured seeds (fig 1). This rate of water uptake per intact seed was unaffected by exposure to far-red light in the presence or absence of germination-promoting chemicals (gibberellic acid, kinetin, or thiourea), but was completed within 15 to 30 minutes in punctured seeds (Haber, unpublished).

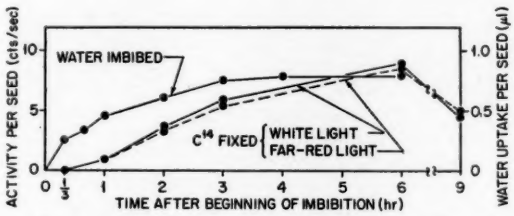


Fig. 1. Fixation of $C^{14}$ from $NaHC^{14}O_3$ and water imbibition. Dry seeds were put into a solution of $NaHC^{14}O_3$ at zero time. Groups of seed were removed and extracted at times indicated.

Distribution of $C^{14}$: Detectable $C^{14}$ was fixed from $NaHC^{14}O_3$ into soluble compounds by intact seeds within the 1st hour of imbibition (fig 1). Extracts from seeds, taken at various times from 90 minutes after the beginning of imbibition until after radicle protrusion, were chromatographed. A radioautograph of the chromatograph of seeds after 90 minutes is reproduced in figure 2. Analysis of extracts after 3 hours imbibition showed that about one third of the total fixed $C^{14}$ appeared in malic acid, and 12 to 15 % in each of the following: glutamine, aspartic acid, citric acid, or glutamic acid. Other labeled compounds were asparagine, serine, glycine, succinic acid, fumaric acid, glyceric acid, and alanine. The over-all qualitative distribution of $C^{14}$ among soluble compounds was similar during all phases of germination, even after protrusion of the radicle. The data suggest that the $C^{14}$ was fixed primarily by carboxylations into organic acids and that the tricarboxylic acid cycle and transamination mechanisms functioned during the earliest phases of germination. The lag in $C^{14}$ fixation as compared with water imbibition does not necessarily imply a lag in $C^{12}$ fixation, but may be attributed to failure of sufficient $C^{14}$ to enter the seed during the 1st 20 minutes of imbibition. Neither the time course of total fixed $C^{14}$ (fig 1) nor the distribution of $C^{14}$ among soluble compounds per seed appeared to be altered by continuous exposure during the 1st 12 hours to far-red light, even though seeds germinated in this

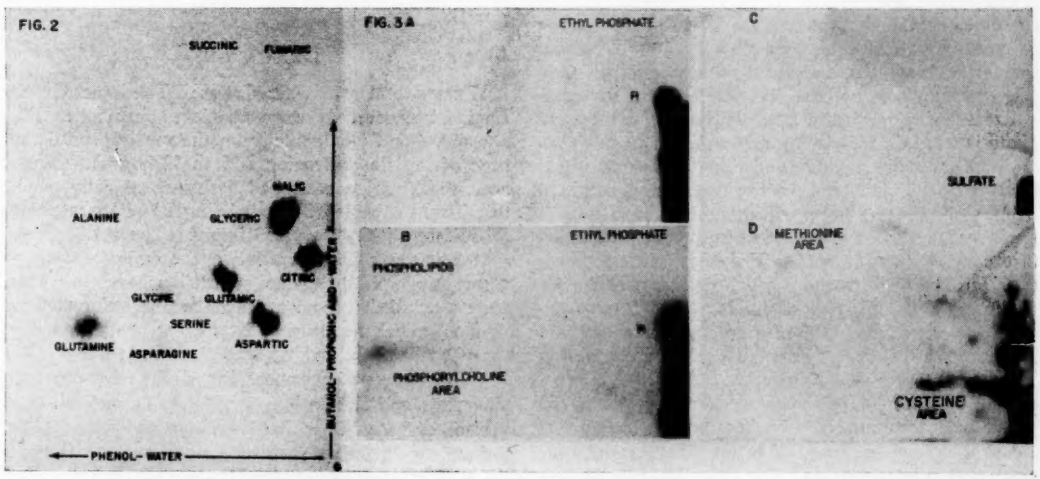


Fig. 2. Radioautogram of chromatograph of extracts from seeds after 90 minutes in $NaHC^{14}O_3$.

Fig. 3. Radioautographs from extracts of seeds that had been in solutions containing tracers for 12 hours. The chromatograms from which the films were made were developed from right to left with phenol : water, and from bottom to top with butanol : propionic acid : water. 3 A. unpunctured seed; with $P^{32}$-orthophosphate; 3 B. punctured seed; with $P^{32}$-orthophosphate; 3 C. unpunctured seed; with $S^{35}$-sulfate; 3 D. punctured seed; with $S^{35}$-sulfate. The chromatograms of 3 A and 3 B were folded parallel to the direction of phenol movement so as to expose both sides of the film. Consequently, the position of the phospholipid spot in 3 B is not indicative of its actual $R_f$ in butanol : propionic acid : water.

way gave 3 % germination after 24 hours under far-red light compared with 85 % germination under white light.

DISTRIBUTION OF $P^{32}$: Intact seeds were germinated in $NaH_2P^{32}O_4$ and extracted at various times through radicle protrusion (15 to 18 hours after beginning imbibition) and into the seedling growth phase. At any time prior to radicle protrusion, the only labeled compounds detected were orthophosphate and ethanol-1-phosphate (fig 3 A). The $P^{32}$-ethyl phosphate could be accounted for by formation of ethyl phosphate from orthophosphate and ethanol as an artifact produced during the extraction procedure, since it could be detected after administration of $P^{32}$-phosphate to extracts of lettuce seeds not previously in contact with the phosphate tracer. Runeckles (14) and Loewenberg, Tolbert, and Haber (unpublished) observed similarly the formation of ethyl phosphate after extraction of various plant tissues with ethanol. Extracts from unpunctured seeds before protrusion were also chromatographed with methanol : formic acid : water. No esterification of $P^{32}$-phosphate was detected on chromatograms developed with this solvent, which we have found to separate orthophosphate from other phosphorus compounds (sugar phosphates, pyrophosphate) that chromatograph near the orthophosphate region in the double solvent system routinely used (Haber and Tolbert, unpublished). Thus, before appearance of the radicle, no $P^{32}$-orthophosphate that could be detected with our chromatographic procedures was esterified by intact seeds. At the time of radicle protrusion, unpunctured seeds attained the capacity to esterify detectable $P^{32}$-orthophosphate from the external solution, and during the subsequent seedling growth phase, activity was detected in many compounds characteristically labeled when $P^{32}$-orthophosphate is administered to intact plants.

In contrast, when punctured seeds were used, esterification of $P^{32}$-orthophosphate was detected within 3 hours after the beginning of imbibition, even though punctured seeds did not germinate any sooner than intact control seeds. Of the $P^{32}$-phosphate esterified, considerable activity corresponded to phosphorylcholine (12) and phospholipids (2). Radioautographs of chromatograms of extracts from intact and punctured seeds after 12 hours in a solution containing $P^{32}$-phosphate are shown in figure 3 A, B. The presence of $P^{32}$-orthophosphate on the chromatograms can probably be accounted for by adsorption of the external solution on surfaces external to the embryo, since the seeds had not been washed so as to quantitatively remove adsorbed $P^{32}$-phosphate. The distribution of tracer in extracts of punctured seeds before radicle protrusion was similar to that obtained from unpunctured seeds after protrusion.

Far-red light exposure in the presence or absence of gibberellic acid, kinetin, or thiourea did not seem to alter either the inability of the seeds to metabolize the tracer or the distribution of $P^{32}$ in extracts of punctured seeds before protrusion.

DISTRIBUTION OF $S^{35}$: Intact seeds that were germinated with $Na_2S^{35}O_4$ and extracted any time prior to radicle protrusion yielded no labeled organic compounds (fig 3 C). Around the time of radicle protrusion, unpunctured seeds attained the capacity to metabolize $S^{35}$-sulfate from the external medium. These results are parallel with those reported above with intact seeds and phosphate tracer.

When punctured seeds were used, $S^{35}$-sulfate was metabolized within 3 hours after beginning of imbibition, suggesting a further parallel between results with sulfate and phosphate tracers. The distribution of $S^{35}$ in extracts of punctured seeds before radicle protrusion was similar to that in unpunctured seeds soon after radicle protrusion. $S^{35}$ was detected in numerous spots, 2 of which corresponded to cysteine and methionine. Radioautographs of chromatograms of extracts from intact and punctured seed after 12 hours in a solution containing radioactive sulfate are shown in figure 3 C, D. The presence of radioactive sulfate on the chromatograms can probably be accounted for by adsorption of the external solution on surfaces external to the embryo.

During the seedling growth phase (followed up to 60 hours after beginning imbibition), the fraction of $S^{35}$ appearing as methionine increased so that methionine had more activity than any other organic compound. The $S^{35}$-methionine from chromatograms of extracts from such seedlings was identified as methionine by elution and cochromatography with *tert*-amyl alcohol : water and with ethanol : glacial acetic acid.

## DISCUSSION

PERMEABILITY OF SEED COATS: The intact Grand Rapids lettuce seeds were unable to metabolize $P^{32}$-phosphate or $S^{35}$-sulphate from the medium until the time of radicle protrusion (15 to 18 hours). However, seeds whose coats had been mechanically punctured were capable of metabolizing $P^{32}$-phosphate and $S^{35}$-sulfate within the 1st 3 hours. The results suggest that the coats surrounding the embryo are impermeable to phosphate and sulfate ions, even in tracer amounts, and that the coats must be ruptured, whether by mechanical puncture or by radicle protrusion, for the phosphate and sulfate tracers to be metabolized.

The apparent impermeability of the seed coats to phosphate and sulfate ions might be alternatively explained by postulating that these ions did penetrate to the embryo, but that they were not metabolized until the time of protrusion. Metabolic changes have been shown to occur within the seeds around the time of protrusion (6), and our data could thus be interpreted to mean that, at the time of radicle protrusion, metabolic processes were activated that in turn resulted in the metabolism of the labeled phosphate and sulfate. According to this latter alternative, we must similarly postulate that the observed metabolism of phosphate and sulfate tracers by pricked seeds before radicle protrusion was occasioned by a more vigorous phosphate

and sulfate metabolism somehow caused by the mechanical puncture. Mechanically punctured seeds, however, did not germinate any sooner than unpunctured controls. This would suggest that the failure of unpunctured seeds to utilize $P^{32}$-phosphate and $S^{35}$-sulfate is indeed caused by failure of the tracers to reach active metabolic sites within the interior of the seeds. This suggestion is supported by experiments cited by Evenari (5), which showed that dinitrophenol stimulated oxygen uptake of Grand Rapids lettuce seeds during the earliest phases of germination. We have confirmed this effect of dinitrophenol by studies using the same experimental conditions and the same batch of seeds used in the tracer studies here reported. $5 \times 10^{-4}$ M 2,4-dinitrophenol, which completely prevented germination of these seeds, increased the average oxygen uptake per 100 intact seeds from 34.1 to 39.1 microliters ($t = 2.70$; d.f. $= 6$; $p = 0.035$) between 4 and 5 hours after the beginning of imbibition. Stimulation of oxygen uptake by dinitrophenol is generally regarded to be a consequence of the greater availability of phosphate acceptors caused by uncoupling of phosphorylation from oxidation (11). Thus it follows that the seeds probably effected phosphorylations during the earliest phases of germination. Failure to esterify $P^{32}$-phosphate under conditions in which the seeds nevertheless effect phosphorylations strongly implies failure of the $P^{32}$ to reach the active sites for phosphorylation. That seed coverings may check the intake of salts ($KNO_3$, NaCl) is also suggested by investigations (reviewed by Crocker and Barton, (4)) performed before the availability of tracers.

The seeds fixed $C^{14}$ within 1 hour from the beginning of imbibition in a buffered $NaHC^{14}O_3$ medium. Since the coats surrounding the embryo were impermeable to phosphate and sulfate ions, it is also possible that bicarbonate ion did not penetrate the seed coats. There is an equilibrium between $C^{14}O_2$ and $NaHC^{14}O_3$ in solution. Moreover, since germinating seeds excrete acid into the medium (4), $HC^{14}O_3^-$ could have been converted to $C^{14}O_2$ at the surface of the seeds. Thus the $C^{14}$ may have reached the embryo in the form of $C^{14}O_2$ rather than as bicarbonate ion. This suggestion is corroborated by the fact that during a 24-hour period the radioactivity of $C^{14}$ in the medium dropped to 2% of initial activity, although the pH did not fall and the $C^{14}$ fixed by the seeds was probably negligible. As a result, the total fixed $C^{14}$ per seed decreased after 6 hours of imbibition, owing to a loss of $C^{14}$ from the medium as well as from the seeds by respiration (fig 1). The observed fixation of $C^{14}$ by the seeds cannot be explained by microbial contamination on the surface of the seeds, since the intact seeds did not metabolize $P^{32}$-phosphate or $S^{35}$-sulfate.

METABOLIC IMPLICATIONS: Poljakoff-Mayber and Evenari (13) have shown that a particulate fraction from Grand Rapids lettuce seedlings is capable of oxidizing a number of substrates of the tricarboxylic acid cycle. However, particulate fractions extracted from untreated seeds before radicle protrusion, or even 1 day after protrusion, showed negligible capacity to oxidize these substrates. Results of our work imply the operation of the tricarboxylic acid cycle as early as 90 minutes after the beginning of imbibition. The pattern of fixation was similar to typical $C^{14}O_2$ dark-fixation patterns (fig 2).

The similarity of distribution of $C^{14}$ among compounds in seeds under white light or far-red light suggests that the effects of photomorphogenic radiation on $CO_2$ fixation are either nonexistent or too subtle for detection by the methods utilized. Similar conclusions were reached by Kunitake et al (10) with respect to $CO_2$ dark fixation during photoperiodic induction in leaves of Kalanchoë.

Generally, the operation of the tricarboxylic acid cycle is linked to the production of high-energy phosphate compounds, which in turn mediate the phosphorylation of many other compounds. The detection of $P^{32}$ in phosphorylcholine and phospholipids within 3 hours after the beginning of imbibition by punctured seeds in a solution containing $P^{32}$-orthophosphate suggests that these synthetic processes are linked very early in the germination process. These results do not support the hypothesis that the block of germination effected by far-red light can be identified with an incapacity of the seeds to effect phosphorylations, since the seeds are still photosensitive much later than the time at which esterification of $P^{32}$-phosphate was first detected in punctured seeds (8). It is not now feasible to defend this last suggestion by the more direct experiment of comparing phosphate esterification in seeds germinated under white light with seeds under far-red light, since puncturing the seeds (necessary for the tracer to reach the embryo) also releases them from far-red light control, as previously described by Evenari and Neumann (7).

Sulfur was detected in sulfur-containing amino acids from punctured seeds after only 3 hours in a solution containing $S^{35}$-sulfate. Thus the mechanisms by which energy is coupled to the endergonic processes of sulfate reduction and phosphate esterification operate during the earliest phases of germination.

## SUMMARY

Grand Rapids lettuce seeds were germinated in solutions containing $C^{14}$-bicarbonate, $P^{32}$-phosphate, or $S^{35}$-sulfate. Extracts from seeds at various times during the course of germination were analyzed by paper chromatography.

$C^{14}$ was detected in soluble compounds as early as 1 hour after the beginning of imbibition. The data suggested that $C^{14}$ was fixed by carboxylations into organic acids and that the tricarboxylic acid cycle and transamination mechanisms functioned during the earliest phases of germination. No difference in the extent or pattern of $C^{14}$ fixation was noted between seeds germinating under white light or under far-red light.

Neither the $P^{32}$-phosphate nor the $S^{35}$-sulfate were metabolized by intact seeds until after radicle protrusion (15 to 18 hours after beginning of imbibition). Punctured seeds, however, metabolized the radioactive phosphate or sulfate within 3 hours after the beginning of imbibition. We concluded that the coats surrounding the embryo are impermeable to phosphate and sulfate ions. Most of the organic $P^{32}$ extracted, whether from punctured seeds before radicle protrusion or from unpunctured seeds after protrusion, was found in phosphorylcholine and phospholipids.

$S^{35}$ was detected in cysteine, methionine, and numerous unidentified compounds, both from punctured seeds before radicle protrusion and from unpunctured seeds after radicle protrusion.

Thus within 3 hours after the beginning of imbibition, punctured seeds could esterify phosphate and reduce sulfate to the level of sulfhydryl.

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# MECHANISMS OF ACTION OF POLYMYXIN B ON CHLORELLA AND SCENEDESMUS[1, 2, 3]


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The investigations of the mechanism of action of an inhibitor of cell growth or development often yield fundamental information about the metabolism of the species under study. The available data on the biochemical constituents of the organisms are commonly insufficient to permit interpretation of experimental results obtained when the inhibitor is introduced. The examination of the role of polymyxin B, an antibiotic which shows strikingly different effects on related species, is no exception. Evidence has been accumulating which indicates that polymyxin B acts to disorganize the cell wall which in turn causes disruption of the osmotic equilibrium of the cell (1, 8, 9, 10). However, other evidence (2, 11, 17) suggests that damage from surface action does not completely explain polymyxin inhibition. Osmotic disruption seems to be but one of several possible effects. Warren et al (17), studying the sensitivity of polymyxin-treated cells to lysozyme, observed that no correlation could be demonstrated between the sensitivity of the organisms to the antibiotic and the lytic response following the addition of the enzyme. Galloway and Krauss (2) showed that galactose can protect against the inhibition of respiration by polymyxin B in 2 species of bacteria.

The present study was undertaken to further examine the role of polymyxin B, especially with regard to the protective effect of galactose on susceptible algae, as well as the mechanism of resistance in normally resistant algae. Such an investigation has


[1] Received September 29, 1958.

[2] This investigation was supported in part by the Rockefeller Foundation and the Office of Naval Research.

[3] Contribution no. 2954 Maryland Agricultural Experiment Station, Scientific Article A 718.

required additional exploration of the metabolism of both kinds of organisms.

## Materials and Methods

Organisms and Media: The algae in these studies included *Chlorella pyrenoidosa* Chick, Van Niel's strain, *Chlorella vulgaris* Beijerinck, Pringsheim's strain, isolated by Emerson, and *Scenedesmus obliquus* Turp. (Kutz), strain WH-50, isolated by Krauss. The several media differed only with respect to the amount of phosphorous and the presence or absence of tris (hydroxymethyl) aminomethane (Sigma 7–9) buffer. The components common to all media were $KNO_3$ (1.00 g/l), $MgSO_4 \cdot 7H_2O$ (0.25 g/l), and micronutrients in the form of inner complex salts of ethylenediaminetetra acetic acid (EDTA) as suggested by Thomas and Krauss (15). The micronutrients included iron (5 ppm), manganese, calcium, copper, cobalt, and zinc (1 ppm each). The "high-phosphate" medium contained, in addition to the common components, $1.64 \times 10^{-3}$ M phosphorous. The "low-phosphate" medium included $1.61 \times 10^{-4}$ M phosphorous and Sigma 7–9 (2 g/l). The "$^{32}$P medium" contained, in addition to the common components, $1 \times 10^{-5}$ M phosphorous and 10 $\mu$c $^{32}$P per $\mu$g of non-radioactive phosphorous. Sigma 7–9 (2 g/l) was also added. The composition of the "$^{32}$P-EDTA medium" was the same, except for the addition of 6 g/l of EDTA. The "phosphorous-free" medium included Sigma 7–9 (2 g/l). The pH of all media was adjusted to 7.0 with HCl.

Manometry: Manometric experiments involving the usual manometric techniques (16), were performed in a standard Warburg apparatus at 25° C. The shaking rate was 125 complete cycles per minute. Vessels were of the 2-side-arm, center-well, venting-plug type. One ml of medium containing a dry weight of cells of about 10 mg was placed in the main compartment of the vessel. The center well contained 0.2 ml of 10 % KOH. One half ml of solution containing various concentrations of polymyxin B was placed in 1 of the side arms. The other was used for the addition of substrate.

Growth: The experimental conditions and equipment have been previously described (2). Briefly, 16-mm culture tubes, containing 5 ml of inoculated medium, were held at a 15° angle on a reciprocating shaker. A bank of fluorescent and incandescent lights supplied an incident illumination between 700 and 1000 ft-c at the surface of the shaker. Temperature was maintained at 25°C.

Phosphoglucose Isomerase: Phosphoglucose isomerase was prepared from rabbit muscle according to the method of Slein (13). The reaction mixture consisted of 0.1 ml of crude enzyme preparation which had been diluted 1 to 4000 with distilled water redistilled in an all pyrex still and 0.4 ml of buffer solution containing various concentrations of the disodium salt of glucose-6-phosphate. The buffer, 0.06 M Sigma 7–9, was adjusted to a pH of 7.0. Incubation was performed at 6° C for 35 minutes. Termination of the reaction was accomplished by addition of 3.5 ml of 8.3 M HCl. Enzyme activity was assayed by determination of fructose-6-phosphate, using the colorimetric method of Roe (12)—viz., 1 ml. of 1 % resorcinol in 95 % ethanol was added and the mixture was kept at 80° C for 10 minutes; then it was cooled in a water bath at 25° C. The color intensity was read in a Bauch and Lomb Spectronic 20 spectrophotometer at a wave length of 540 m$\mu$.

Uptake of $^{32}$P: Algae were cultured in 35 ml of either low-phosphate or high-phosphate medium in 75 ml test tubes in a 25° C water bath. The cultures were bubbled with air enriched with 5 % $CO_2$. Light was supplied from 2 sides by fluorescent lamps, each group of which gave an irradiance of approximately 1500 ft-c at the surface of the culture tubes. The cultures were grown to an optical density between 0.75 and 1.0 measured at a wave length of 560 m$\mu$. Cells harvested at this time were predominately large, mature light cells. In order to obtain predominately starved and young dark cells, cultures were placed in the dark for 24 hours before use. The term "light" cells refers to cells which are at the end of the developmental cycle and are nearly ready to reproduce vegetatively by dividing into daughter cells. In contrast "dark" cells are young cells which may be formed in the dark by division of light cells. They remain small and physiologically "young" if kept in the dark without an energy source.

From this point on, 1 of 2 procedures was employed, depending upon the length of time cells were to be exposed to $^{32}$P. For a treatment of 15 minutes or less, usually the following method was used: The cells were removed from the medium by centrifugation for 2 minutes at 675 × G in 5-ml conical tubes in an angle-head Sargent centrifuge. Cells were washed once, resuspended in phosphorous-free medium, and dispensed into a sintered glass funnel fitted with a millipore filter disc (HA plain white, 47 cm in diameter). Aliquots of $^{32}$P-EDTA medium were then thoroughly mixed with the cell suspension in the funnel. (EDTA was included to prevent the precipitation of phosphate salts on the membrane observed in preliminary studies—see Results.) After specified periods of time, the medium was removed by suction through the filter, leaving a thin layer of radioactive cells. The filter (with cells) was removed and fastened with rubber cement to the absorbent pad provided for each filter by the manufacturer. When the cells were dry, their radioactivity was measured on a Tracerlab autoscaler with an end-window Geiger-Mueller tube. Alternatively, if cells were to be in contact with $^{32}$P for longer than 15 minutes, $^{32}$P-EDTA medium was usually added directly to the centrifuge tubes. After a given time, the cells were centrifuged from the medium, resuspended in phosphorous-free medium, and an aliquot was placed in the center of a metal planchet, dried, and assayed as before.

SUGAR-PHOSPHATE ESTERS—BIOSYNTHESIS AND CHROMATOGRAPHY: Algae were cultured in 35 ml of low-phosphate medium under the same conditions as those described above in the studies of $^{32}P$ uptake. When the optical density of the cultures was between 0.75 and 1.0, they were placed in complete darkness for 24 hours at the end of which time they were harvested by centrifugation, washed once in phosphorous-free medium, and resuspended in 15 to 20 ml of $^{32}P$ medium to give a calculated optical density of 3.5. After 10 minutes the medium was removed by centrifugation and the cells were resuspended in phosphorous-free medium. They were retained 5 minutes in this medium to allow for equilibration of $^{32}P$ with the $^{31}P$ in the cells. The suspension was again centrifuged and the cells resuspended in phosphorous-free medium or in phosphorous-free medium to which had been added 0.05 M concentrations of glucose, galactose, or fructose. Aliquots were taken after specified periods, the medium was removed, and the cells were extracted. Extraction was accomplished by resuspending the cells in distilled water (1 ml for each ml of $^{32}P$ medium used) and by placing the cell suspension in a 100° C water bath. After 1 minute they were rapidly returned to room temperature in another water bath after which the cells were removed by centrifugation. The cell-free extracts were used immediately or stored overnight at 1° C.

The extract was applied as a spot 1 3/4 inches from 1 edge of large sheets (18 1/4 in. × 22 1/2 in.) of Whatman no. 3 paper pre-washed in 95 % ethanol : glacial acetic acid : and water (2 : 1 : 1, v/v/v). Usually, several applications of 5 lambda each were made, the spot being dried by a stream of air at room temperature between applications. Five-lambda quantities of 0.5 M solutions of each of 4 known compounds—glucose-1-phosphate, glucose-6-phosphate, galactose-1-phosphate, and galactose-6-phosphate—were applied to the same spot. A partial separation of the extract into its several components was achieved by means of a descending movement in the long dimension of the paper with the solvent—isobutyric acid : ammonium hydroxide : and water (57 : 4 : 39, v/v/v)—slightly modified from Kilgour et al (6). A standard commercial chromatographic chamber was used for this 1st separation. After about 24 hours (several hours after the front had moved off the end of the paper) the papers were removed and dried in a current of air at room temperature for 24 hours or more. An 8 1/4 inch wide strip, which included the 4 phosphate esters noted above, was cut across the width of each sheet. For development in the 2nd dimension, the sheets were oriented so that the phosphorous compounds, located 1 3/4 inches from 1 edge of the original sheets, were at the bottom of the 8 1/4 × 18 1/4 inch sheets which remained. The spots were further resolved by the use of a 2nd solvent—methanol : water (7 : 3, v/v), containing 1 % sodium tetraborate. Movement of the solvent was ascending and in a perpendicular direction to that of the 1st solvent. The chambers for the ascending development were 2 cylindrical pyrex jars, 18 inches tall and 12 inches in diameter, fitted with glass covers which were held in place with weights and sealed to the jar with silicone grease. The chambers were fitted with 4 glass rods fixed 3/4 inch from the top of the cylinder and 1 1/2 inches apart. The solvent was placed in the bottom of the chambers and the papers were hung from the glass rods so that they were immersed about 1/4 inch into the solvent. After about 12 hours, when the solvent front had nearly reached the top of the papers, they were removed, dried as before, and placed next to x-ray film in Kodak x-ray exposure holders. After several days of exposure, depending on the activity present, the films were removed and developed. The chromatograms were sprayed with Hanes and Isherwood's reagent (2), dried for several minutes at 85° C, hydrated with steam, and exposed to $H_2S$. With this procedure the authentic spots of glucose-1-phosphate and galactose-1-phosphate became blue-green, those of glucose-6-phosphate and galactose-6-phosphate became blue, and the background remained nearly white. (From one-dimensional chromatograms made with each solvent, the $R_f$ values of each of the known compounds had been established, enabling their identification on the two-dimensional chromatograms.) (Table I.) Radioactive spots of phosphate-compounds from the extracts of the cells were of such low concentration that no blue coloration resulted from their presence. The spots corresponding to the 4 known compounds were identified on the radioautogram. Semi-quantitative determinations of these compounds in the extract were accomplished by assaying the radioactivity in the corresponding spots. They were cut from the chromatogram and evaluated in a windowless flow counter.

TABLE I

$R_f$ VALUES OF AUTHENTIC PHOSPHATE COMPOUNDS

| COMPOUND | SOLVENT NO. 1* | SOLVENT NO. 2** |
|---|---|---|
| Fructose-1,6-diP | 0.22 | ... |
| Fructose-6-P | 0.24 | ... |
| Galactose-6-P | 0.31 | 0.34 |
| Glucose-6-P | 0.32 | 0.26 |
| Glucose-1-P | 0.37 | 0.37 |
| Galactose-1-P | 0.37 | 0.29 |
| Ortho phosphate | 0.51 | 0.28 |

* Isobutyric acid : ammonium hydroxide : water (57 : 4 : 39, v/v/v).

** Methanol : water (7 : 3, v/v).

## RESULTS

Polymyxin B has been shown to have detrimental effects on the growth and respiration of *Chlorella pyrenoidosa*, whereas *C. vulgaris* and *Scenedesmus obliquus* are unaffected by far higher concentrations (2). Since previous work (2) has also indicated that susceptible organisms may become resistant when grown on galactose, experiments were performed to determine if the sugar would function in such a pro-

tective role in the case of *Chlorella pyrenoidosa*. Before proceeding with relatively long-term experiments involving polymyxin B in cultures, however, it seemed desirable to determine if the antibiotic would be inactivated under the environmental conditions of the culture apparatus. Five-ml aliquots of inorganic medium containing 5, 10, 20, or 40 ppm of polymyxin B were dispensed into 16-mm test tubes and placed on the shaker. After 26 days a similar series of media was again prepared and both sets were inoculated with 0.1 ml aliquots of a culture of *C. pyrenoidosa*, a susceptible organism. The results are shown in table II. The data indicate that under these conditions the antibiotic is inactivated with time. In order to establish more precisely the required time for deactivation, growth measurements were made for a longer period of time in another series of cultures using freshly prepared media containing polymyxin B (table III). Growth eventually began in the tubes containing 20 ppm polymyxin (14 days) and still later in those with 40 ppm (29 days). Even after growth was visually evident, the rate was always somewhat slower than that in medium with no polymyxin B. Probably growth commenced prior to complete destruction of the antibiotic. The growth rate returned to normal in a few days—a fact which would seem to substantiate this conclusion. If growth began in the presence of a low concentration of polymyxin B, less than 5 ppm, it seemed possible that these cells had acquired resistance by either genetic or physiological mechanisms. To determine if this were the case, a culture which had begun to grow in the presence of 40 ppm (after 31 days) was washed and used as inoculum in media containing 0, 5, 10, 20, and 40 ppm polymyxin B. A comparison of the data from this type of experiment, table IV, with that obtained under similar conditions using cells never exposed to polymyxin B prior to this experiment, table III, indicates that resistance was attained although not nearly so much as that reported for *Escherichia coli* (14). Further experiments revealed that resistance could not be further increased by continual growth in low concentrations of polymyxin B.

During these experiments, 2 effects of polymyxin B were observed in addition to its effect on growth: 1) cultures became completely bleached in 4 to 6 hours in the presence of concentrations sufficient to cause inhibition of growth; and, 2) release of daughter cells from mother cells was inhibited.

TABLE II

OPTICAL DENSITIES OF CHLORELLA PYRENOIDOSA, GROWN IN FRESH AND OLD MEDIA CONTAINING VARIOUS CONCENTRATIONS OF POLYMYXIN B

| DAYS AFTER INOCULATION | FRESH MEDIUM POLYMYXIN B, (PPM) | | | | | 26-DAY-OLD MEDIUM POLYMYXIN B, (PPM) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 5 | 10 | 20 | 40 | 0 | 5 | 10 | 20 | 40 |
| 0 | 0.07 | 0.07 | 0.07 | 0.07 | 0.07 | 0.07 | 0.07 | 0.07 | 0.07 | 0.07 |
| 1 | 0.35 | * | * | * | * | 0.36 | 0.31 | 0.30 | 0.13 | 0.09 |
| 2 | 0.77 | * | * | * | * | 0.76 | 0.72 | 0.71 | 0.25 | 0.09 |

* No growth.

TABLE III

EFFECT OF TIME ON THE DECOMPOSITION OF POLYMYXIN B, AS INDICATED BY THE GROWTH OF CULTURES OF CHLORELLA PYRENOIDOSA

| DAYS AFTER INOCULATION | POLYMYXIN B, PPM | | | | |
|---|---|---|---|---|---|
| | 0 | 5 | 10 | 20 | 40 |
| 0 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| 1 | 0.43 | * | * | * | * |
| 2 | 0.78 | * | * | * | * |
| 3 | 1.05 | * | * | * | * |
| 4 | 1.25 | 0.20 | * | * | * |
| 5 | ** | 0.62 | * | * | * |
| 7 | ** | 1.10 | 0.20 | * | * |

* No growth.

** Measurements discontinued.

TABLE IV

OPTICAL DENSITIES OF POLYMYXIN-CONDITIONED CULTURES OF CHLORELLA PYRENOIDOSA GROWN IN MEDIA CONTAINING VARIOUS CONCENTRATIONS OF POLYMYXIN B*

| DAYS AFTER INOCULATION | POLYMYXIN B, PPM | | | | |
|---|---|---|---|---|---|
| | 0 | 5 | 10 | 20 | 40 |
| 0 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| 1 | 0.44 | 0.30 | 0.26 | ** | ** |
| 2 | 0.80 | 0.72 | 0.57 | ** | ** |
| 3 | 1.10 | 0.92 | 0.85 | ** | ** |
| 4 | 1.30 | 1.20 | 1.15 | ** | ** |
| 5 | *** | *** | *** | ** | ** |
| 6 | *** | *** | *** | ** | ** |
| 7 | *** | *** | *** | ** | ** |

* The inoculum was from a culture grown in medium containing 40 ppm of polymyxin B.

** No growth.

*** Measurements discontinued.

The bleaching phenomenon may be explained by assuming that the cells had been killed, subsequent chlorophyll break-down occurring rapidly under the relatively high light intensity of these experiments. Bleaching did not occur in the dark—a fact which also would seem to indicate that it was not a direct effect of the inhibitor. However, bleached cultures were eventually capable of chlorophyll formation and renewed growth. Whether the entire population was temporarily in a resting state, capable of growth at a future time, or whether most cells were killed and the renewed growth of the culture originated with a relatively few viable cells was a problem worth resolving. Inasmuch as it proved impossible to differentiate microscopically between viable and nonviable cells, á culture procedure was employed.

Aliquots of 0.5-ml of cell suspensions, which had been exposed for 8 hours to 1, 10, 100, or 1000 ppm of polymyxin B, were plated in an inorganic agar medium in Petri dishes. The cells which had been in 1 ppm of polymyxin B produced $5.75 \times 10^6$ colonies per plate, the aliquot from 10 ppm produced 63, that from 100 ppm produced 1, and that from 1000 ppm produced none. After 24 hours in contact with polymyxin B, 0.5-ml aliquots were again removed and plated. Colony production was reduced to $1.29 \times 10^6$ for the 1 ppm level, 3 for the 10 ppm, and none for the 100 or 1000 ppm levels. Thus it seems evident that: 1) new growth starts from a few remaining survivors and not from renewed growth of the entire culture; and, 2) the bleached appearance of the culture results from the destruction of chlorophyll after death of most cells.

The 2nd observation was that abnormally large cells appear in cultures of *Chlorella pyrenoidosa* treated with polymyxin B. The antibiotic has been reported to produce thread-like cells of *Escherichia coli* (11). In the alga, cell division seemed to progress normally, but subsequent disruption of the mother cell wall failed to take place. In some cases, 64 and occasionally 128 daughter cells were inside of 1 mother cell wall (fig 1). The amount of old cell wall material in the culture was also abnormally high, another indication that lysing of the cell wall was slower than normal. A similar inhibition of disruption of the cell wall may cause the abnormally long cells of *E. coli*.

FIG. 1. A photomicrograph of *Chlorella pyrenoidosa* grown for 24 hours in an inorganic medium containing 15 ppm of polymyxin B (magnified × 5000).

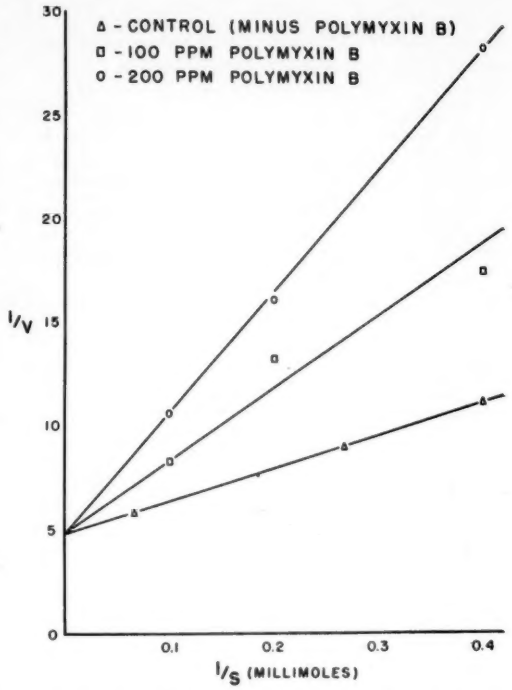


FIG. 2. A double-reciprocal plot of the effect of polymyxin B on the activity of phosphoglucose isomerase obtained from rabbit muscle. The reaction mixture consisted of 0.1 ml of enzyme preparation which had been diluted 1 to 4000 with double-distilled water, and 0.4 ml of buffer solution, 0.06 M Sigma 7–9, adjusted to a pH of 7.0. Incubation was performed at 6° C for 35 minutes. $1/v$ = inverse of the optical density of the product of the reaction, fructose-6-phosphate, assayed according to the method of Roe. $1/S$ = inverse of the concentrations of the substrate, glucose-6-phosphate. Each point is an average of 4 determinations.

Experiments designed to evaluate the effectiveness of galactose against inhibition of growth by polymyxin B were undertaken. Using medium containing 0.5 % galactose and polymyxin B concentrations of 5, 10, 20, and 40 ppm, it was found that at first no growth was obtained in the presence of any amount of polymyxin, whether galactose was present or not. However, as the time for sufficient deactivation of each higher concentration of the inhibitor elapsed, growth always began 2 or 3 days earlier in the respective medium with galactose.

For an organism to be able to utilize galactose in the presence of an inhibitor which prevents its use of glucose, it was logical to postulate that 1 of several enzyme systems was being damaged. It was reasoned that phosphoglucose isomerase was the most likely

enzyme to be susceptible. Experiments showed that polymyxin B indeed has an inhibitory effect on this enzyme. In order to determine the type of inhibition being effected the data were plotted on double-reciprocal axes, after the method of Lineweaver and Burk (7) (fig 2). These data indicate clearly that inhibition is of the competitive type.

In view of the identification of a probable site of inhibition in the glycolytic pathway, it became imperative to examine the compounds formed during the metabolism of the algae. In order to study differences in the utilization of carbohydrate substrates in the presence and absence of polymyxin B, cells were exposed to radioactive phosphorous. Phosphorylated intermediates within the cells were thus made radioactive, and any change in $^{32}P$ distribution could be followed by tracer techniques.

Experiments were performed with *C. pyrenoidosa* to determine the conditions under which $^{32}P$ uptake would occur. For very short term exposures, cell separation from the media by means of a millipore filter seemed most promising. However, it was found that when $^{32}P$ medium was passed through the filter, about 20 % of the total activity was retained by the filter. Perhaps a precipitate of $^{32}PO_4$ and magnesium was being formed which was too small to be visible but too large to pass through the filter. If the $^{32}P$ were placed in distilled water, about 10 % of the activity still remained in the filter. Furthermore, placing cells in distilled water for even a few minutes had detrimental effects on the cells, as evidenced by their release of significant amounts of $^{32}P$ soon after it had been absorbed. Consequently, a chelating agent, EDTA, was incorporated into the medium. It was found that 6 g of $Na_2$ · EDTA per liter was sufficient to prevent more than 1.5 % $^{32}P$ retention on the filter.

A comparison of the uptake of $^{32}P$ by mature light cells and young dark cells is shown in figure 3. It was later observed, by chromatography of cell extracts, that large amounts of metaphosphate are formed in cells grown in medium containing the usual amount of phosphate, referred to in this paper as high-phosphate medium. This polymer not only uselessly accounts for a major portion of the activity, but it also tends to make chromatographic separation of compounds containing $^{32}P$ more difficult. It was reasoned that storage of this "excess" phosphate might be eliminated by the use of a medium with a very low phosphate content. Experiments with all 3 species of algae indicated that growth was normal and sufficient when the medium contained $1.61 \times 10^{-4}$ M phosphorous. This is in agreement with the work of Kamen and Gest (5). Figure 4 shows that the uptake of $^{32}P$ by cells which had been grown in low-phosphate medium was more rapid and more nearly complete than that by cells grown in high-phosphate medium. For this reason, and because of the elimination of metaphosphate, as later shown by chromatography, cells were routinely cultured in low-phosphate medium prior to exposure to $^{32}P$.

It was observed that *C. pyrenoidosa* cells containing $^{32}P$ rapidly released large percentages of it when

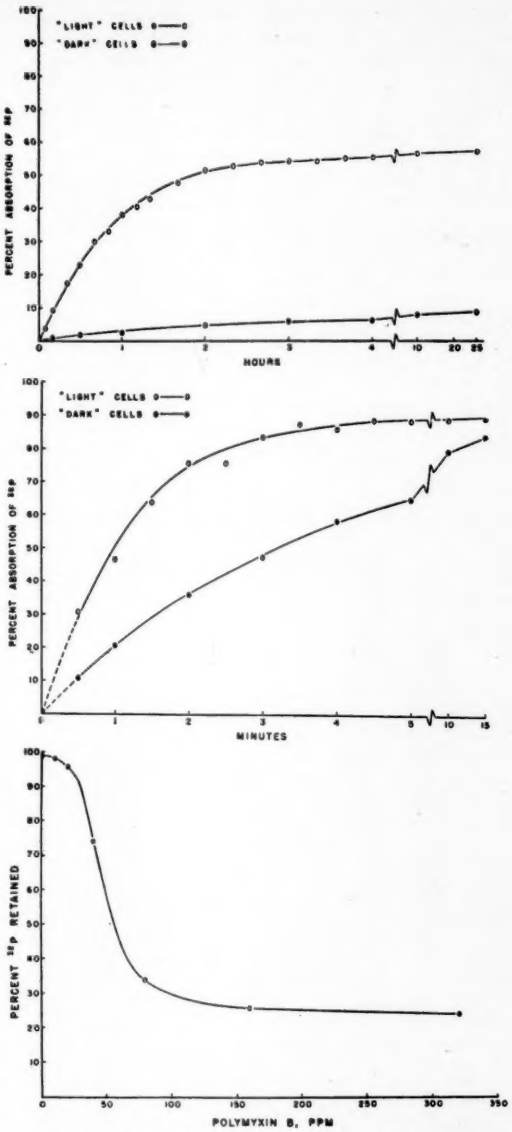


FIG. 3 (*top*), FIG. 4 (*center*). Absorption of $^{32}P$ in the dark by *Chlorella pyrenoidosa*. Figure 3 shows absorption by cells which had been grown in media containing $1.64 \times 10^{-3}$ M phosphorous; Figure 4 shows absorption by cells which had been grown in media containing $1.61 \times 10^{-4}$ M phosphorous. "Light" cells were from a lighted culture; "dark" cells were from a culture darkened for 24 hours prior to exposure to $^{32}P$. The medium in which the algae were exposed contained 10 $\mu$c $^{32}P$ per $\mu$g of $^{31}P$, the latter being at a concentration of $1 \times 10^{-5}$ M.

FIG. 5 (*bottom*). The percent of $^{32}P$ retained in cells of *Chlorella pyrenoidosa* after 15 minutes in media containing various concentrations of polymyxin B.

placed in medium containing polymyxin B. The amount of $^{32}P$ lost varied with concentration of inhibitor, as shown in figure 5. Inspection of radioautographs of the material lost from the cells (fig 6) revealed that many compounds were involved. Previous work (2) showed that the rate of respiration of glucose by *C. pyrenoidosa* was temporarily depressed in the presence of 80 ppm or less of polymyxin B. Within 2 hours, however, the rate was approximately that of untreated cells. It is to be expected that upon resumption of respiration, $^{32}P$ lost from the cells would be resorbed. In order to determine if this were the case, 2 treatments were executed. In 1, cells containing $^{32}P$ were placed in phosphorous-free medium to which various concentrations of polymyxin B, and 0.05 M glucose had been added. In the 2nd, similar cells were exposed to light and bubbled with 5 % $CO_2$-in-air. The amount of $^{32}P$ released from the cells in 15 minutes was the same for all treatments at any given concentration of polymyxin B. However, at the end of 2.5 hours, significant amounts of $^{32}P$ had been resorbed, especially at the lower concentrations of polymyxin B.

The resistant species, *C. vulgaris* and *S. obliquus*, grown on low-phosphate medium and given 24 hours of darkness were found to absorb between 90 and 95 % of the $^{32}P$ from $^{32}P$-medium in 10 minutes. Cells containing $^{32}P$, when resuspended in phosphorous-free medium, released only about 1 % of their $^{32}P$ in the presence or absence of polymyxin B. Radioautograms were prepared of the extracts of the 3 species of algae after they had been placed in phosphorous-free media containing 0.05 M concentrations of glucose, galactose, or fructose for 30 or 90 minutes. Similar radioautograms were made of extracts from the 2 resistant species which had been exposed to 320

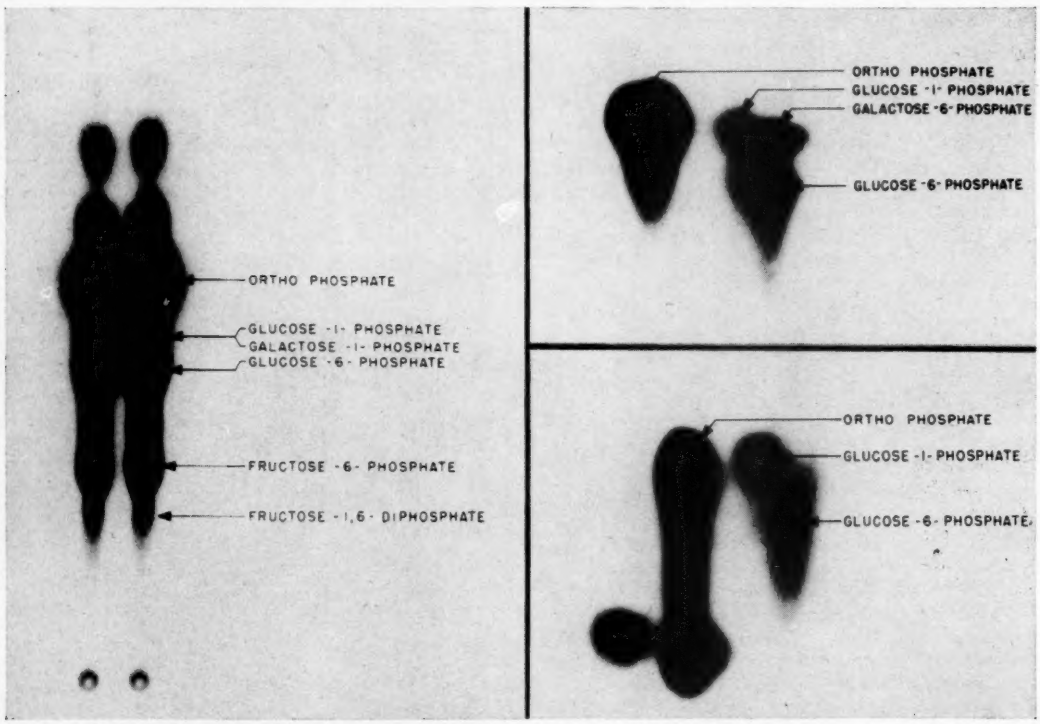


FIG. 6 (*left*). A radioautogram of a filter paper chromatogram showing the $^{32}P$ radioactivity of compounds released from *Chlorella pyrenoidosa* after 15 minutes exposure to 320 ppm of polymyxin B. The cells had been previously suspended for 10 minutes in medium containing $1 \times 10^{-5}$ M $^{31}P$ and 10 $\mu$c $^{32}P$ per $\mu$g of $^{31}P$. The origin is at the bottom. The solvent was isobutyric acid : ammonium hydroxide : water (57 : 4 : 39, v/v/v), 24 hours.

FIG. 7 (*top, right*), FIG. 8 (*bottom, right*). Radioautograms of two-dimensional paper chromatograms showing the $^{32}P$ radioactivity of compounds in water extracts of *Scenedesmus obliquus* after 30 minutes utilization of glucose (fig 7) and of *Chlorella pyrenoidosa* after 30 minutes utilization of fructose (fig 8). The cells had been previously suspended for 10 minutes in medium containing $1 \times 10^{-5}$ M $^{31}P$ and 10 $\mu$c $^{32}P$ per $\mu$g of $^{31}P$. The origin, removed during development in the 2nd dimension, lies off the paper to the lower right. The solvents were: 1st (across—right to left) isobutyric acid : ammonium hydroxide : water (57 : 4 : 39, v/v/v), 24 hours; 2nd (up) methanol : water (7 : 3, v/v) containing 1 % sodium tetraborate, 12 hours.

TABLE V

PERCENTAGE OF THE $^{32}P$ IN CHLORELLA PYRENOIDOSA 2.5 HOURS AFTER TREATMENT WITH VARIOUS CONCENTRATIONS OF POLYMYXIN B *

| POLYMYXIN B, PPM | % $^{32}P$ IN THE CELLS ** | |
|---|---|---|
| | DARK + GLUCOSE *** | LIGHT — GLUCOSE |
| 0 | 96.7 | 96.9 |
| 20 | 98.1 | 97.2 |
| 40 | 86.0 | 88.8 |
| 80 | 48.8 | 17.0 |
| 160 | 38.2 | 11.0 |
| 320 | 19.4 | 8.2 |

* Light-grown cells labeled with $^{32}P$ were treated with the 5 concentrations of polymyxin B. In 15 minutes all but 9.8 % of the $^{32}P$ was lost from the cells regardless of the concentration of polymyxin B.

** This represents the $^{32}P$ remaining in the cells plus that resorbed during 2.5 hours.

*** 0.05 M.

ppm polymyxin B for 1 hour prior to addition of substrate. Examples of these radioautograms are shown in figures 7 and 8. The amounts of glucose-1-phosphate, glucose-6-phosphate, galactose-1-phosphate, and galactose-6-phosphate present in the cells after 30 minutes of utilization of substrates, as revealed by their radioactivity, are shown in table VI.

## DISCUSSION AND CONCLUSIONS

It appears that polymyxin B can injure or destroy certain organisms in at least 2 ways. It may either disrupt the differential permeability of the cell membrane so that cell components are lost, or it can act against specific enzymes essential to metabolism. In regard to inhibition at the enzyme level, it is apparent that polymyxin B prevented the formation of fructose-6-phosphate from glucose-6-phosphate by inhibition of phospho-glucose isomerase. Consequently, resistance requires 1st a membrane which withstands

TABLE VI

COUNTS OF $^{32}P$ PER MINUTE IN FOUR HEXOSE-PHOSPHATE ESTERS IN WATER EXTRACTS OF SCENEDESMUS OBLIQUUS, CHLORELLA VULGARIS AND C. PYRENOIDOSA AFTER UTILIZING SUBSTRATES FOR 30 MINUTES *

| SUBSTRATE | HEXOSE PHOSPHATE ESTER | UNTREATED | | | TREATED** | |
|---|---|---|---|---|---|---|
| | | SCENEDESMUS OBLIQUUS | CHLORELLA VULGARIS | CHLORELLA PYRENOIDOSA | SCENEDESMUS OBLIQUUS | CHLORELLA VULGARIS |
| None | G-1-P*** | 100 (25)‡ | 151 (8) | 0 | 48 (5) | 426 (10) |
| | Ga-1-P† | 0 | 172 (8) | 175 (26) | 218 (22) | 527 (13) |
| | G-6-P | 295 (75) | 1682 (80) | 501 (74) | 711 (73) | 3010 (74) |
| | Ga-6-P | 0 | 90 (4) | 0 | 0 | 107 (3) |
| Glucose | G-1-P | 180 (24) | 710 (6) | 0 | 297 (7) | 837 (9) |
| | Ga-1-P | 0 | 2619 (22) | 362 (29) | 777 (18) | 2400 (26) |
| | G-6-P | 385 (51) | 8370 (71) | 887 (71) | 3354 (75) | 6128 (65) |
| | Ga-6-P | 188 (25) | 42 (1) | 0 | 0 | 0 |
| Fructose | G-1-P | 127 (21) | 395 (7) | 0 | 192 (11) | 427 (9) |
| | Ga-1-P | 0 | 1578 (28) | 199 (32) | 306 (18) | 1510 (32) |
| | G-6-P | 466 (79) | 3564 (63) | 419 (68) | 1250 (71) | 2723 (59) |
| | Ga-6-P | 0 | 82 (2) | 0 | 0 | 0 |
| Galactose | G-1-P | 194 (33) | 2780 (34) | 54 (8) | 289 (11) | 837 (16) |
| | Ga-1-P | 0 | 1188 (14) | 242 (38) | 795 (29) | 1811 (34) |
| | G-6-P | 268 (46) | 3861 (47) | 342 (54) | 1662 (60) | 2631 (50) |
| | Ga-6-P | 119 (21) | 409 (5) | 0 | 0 | 0 |

* Extracts were at 100° C for 1 minute.

** Treatment consisted of exposure to 320 ppm of polymyxin B for 1 hour prior to utilization of substrate.

*** Glucose-1-phosphate.

† Galactose-1-phosphate.

‡ Numbers in parentheses are percentages of total activity of the compounds indicated.

attack, and 2nd, a sequence of enzymes capable of yielding energy to the organisms in the absence of phosphoglucose isomerase. In the case of the resistant species, *Chlorella vulgaris* and *Scenedesmus obliquus*, the evidence indicates that this 2nd requirement has been met by the utilization of galactose-6-phosphate in the synthesis of fructose-6-phosphate, or intermediates below this in the Embden-Myerhof sequence (fig 9). The "by-way" proposed involves first the mutation of galactose-1-phosphate to galactose-6-phosphate under the influence of a phosphogalacto-mutase. The next step in the sequence almost certainly is the conversion of galactose-6-phosphate by means of a phosphogalactose isomerase to tagatose-6-phosphate (4). One of 3 subsequent transformations must occur: 1) tagatose-6-phosphate could be converted to fructose-6-phosphate by means of a phosphotagatose epimerase; 2) the diphosphate ester of tagatose could be converted to fructose-1,6-diphosphate under the influence of a diphosphotagatoisomerase; or, 3) tagatose-1,6-diphosphate itself could be split by means of an aldolase to yield the 2 triose esters. No evidence yet exists which enables a choice among these 3 possibilities of re-entry into the classical glycolytic pathway.

The ability to circumvent a block in the glycolytic pathway in the manner suggested accounts for the protection afforded by galactose in the case of certain otherwise susceptible organisms incapable of obtaining energy from glucose via galactose. This also accounts for the synthesis of galactose-6-phosphate in resistant organisms. The disappearance of galactose-6-phosphate from resistant organisms subsequent to exposure to polymyxin B is interpreted as evidence of increased utilization of this ester under this condition. Simultaneous increase in galactose-1-phosphate indicates that its mutation (the shift of the phosphate moiety from the 1 to the 6 position) is the rate-limiting step in this sequence.

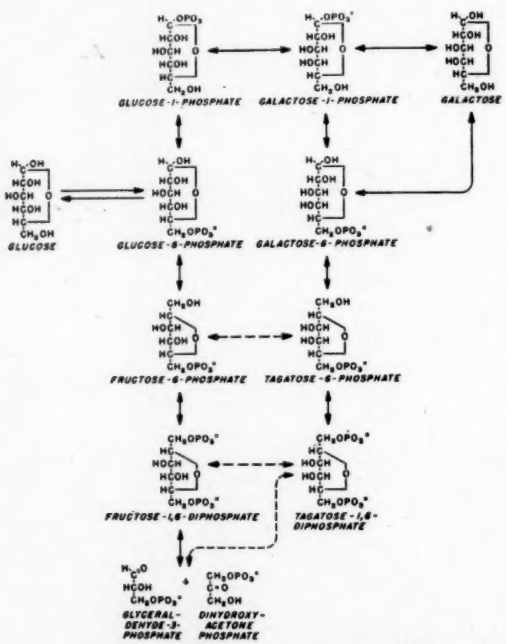


FIG. 9. The sequence of hexose intermediates of an alternate pathway in relation to the classical glycolytic scheme.

Bacteria which gained protection from inhibition of polymyxin B by utilization of galactose (2) are examples of organisms which meet 1 requirement for resistance, i.e., a membrane not subject to disorganization, but not the other, i.e., a method of obtaining energy in the absence of phosphoglucose isomerase, except when supplied with galactose. These species evidently lack at least 1 of the enzyme systems involved in the sequence between glucose-6-phosphate and galactose-6-phosphate.

## SUMMARY

A study was made of the effects of the antibiotic, polymyxin B, on unicellular green algae. The growth and respiration of *C. pyrenoidosa* are inhibited by 5 to 10 ppm of polymyxin B, whereas *C. vulgaris* and *Scenedesmus obliquus* are unaffected. Previous work with susceptible bacteria showed that their rate of respiration of galactose remained unchanged upon exposure to polymyxin B. Lack of a similar protective effect from glucose gave evidence of a new pathway of galactose utilization.

Investigation revealed 2 convergent lines of evidence which implicate phosphoglucose isomerase as the enzyme in glycolysis which is blocked by polymyxin B. First, a direct examination of the effect of the antibiotic on the extracted enzyme showed an inhibition of the competitive type. Second, galactose-6-phosphate is synthesized by the resistant organisms, *Chlorella vulgaris* and *Scenedesmus obliquus*. Further transformations of galactose-6-phosphate would permit re-entry into glycolysis at a point beyond glucose-6-phosphate. A sequence of transformations has been hypothesized: 1) Galactose becomes phosphorylated on carbon-6; 2) a shift in the positions of the ring results in the formation of a ketose, tagatose-6-phosphate; and, 3) the addition of a 2nd phosphate radical yields tagatose-1,6-diphosphate. Re-entry into glycolysis could result from: 1) Conversion of tagatose-6-phosphate to fructose-6-phosphate; 2) conversion of tagatose-1,6-diphosphate to fructose-1,6-diphosphate; or, 3) the splitting of tagatose-1,6-diphosphate into the triose phosphate esters, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.

Another striking aspect of polymyxin B is its ability to disorganize the cellular membrane. The leakage of phosphorous from cells of the susceptible *Chlorella pyrenoidosa* is approximately 80 % after 15 minutes of exposure to polymyxin B.

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# ON THE OCCURRENCE OF FREE GALACTURONIC ACID IN APPLES AND TOMATOES [1, 2]


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Changes in amounts and kinds of pectic substances have long been associated with the ripening process in fruits (12). However, the uronic acids which are characteristic constituents of these and many other plant polysaccharides are not generally considered to occur in free state. Nevertheless, several reports of their occurrence in sound fruits have appeared recently (1, 2, 4, 9, 15, 18, 19). Since most of these reports were based on colorimetric assay methods without characterizing the particular uronic acid concerned, we wish to report here some more precise indications and a few quantitative data on changes with ripeness.

## Materials and Methods

The fruits used in this study were selected from the replicate samples prepared for the study of their polysaccharides (20). They were picked as both unripe and ripe, with the latter allowed to become overripe in the laboratory. Fruits with apparent signs of rotting were discarded.

About 1000 g of the fruit were sliced into boiling 2-propanol (99 %) in an amount sufficient to give a final alcohol concentration of about 70 %. The filtered solutions were reduced to a syrup on a steam bath and stored in the deep freeze till analyzed.

Isolation of the Fruit Acids: The acids in the syrups were freed of neutral sugars and cations by passing them through a 2-step deionization column in a procedure mainly following Winkler (18, 19) and Mills (15). The 1st step was Amberlite IR-120 (beads) in the hydrogen form. The 2nd step was Duolite A-4 (smaller than 40 mesh) in the free base form. Blanks and standards were run as recommended by Winkler. The anion resin was eluted with 20 % formic acid followed by water. Some pigment appeared in the acid eluates of the unknowns.

Paper Chromatography: Uronic acids are more difficult to separate than the corresponding


[1] Received October 1, 1958.

[2] Published as Miscellaneous Paper no. 317, with the approval of the Director of the Delaware Agricultural Experiment Station.

hexoses. Most acidic solvents do not resolve the uronic acids, while in pyridine mixtures they frequently form double spots. However, we have been able to use (table I) the mixture of Masamune and Maki (13) (*n*-butanol : pyridine : water; 7 : 7 : 6 V/V) by treating Whatman no. 1 paper with 0.5 % ammonium pectate and drying prior to use (see also (16)). Descending chromatography was used. Fischer and Dörfel (8) successfully chromatographed the uronic acids more simply in a mixture of ethyl-acetate : pyridine : acetic acid : water (5 : 5 : 1 : 3). We find the addition of a little acetic acid to a variety of butanol (etc.) : pyridine : water mixtures precludes the formation of double spots by uronic acids. A mixture of *n*-butanol : pyridine : water (6 : 4 : 3 V/V) was used for hexoses and pentoses (11); the addition of 1 part of acetic acid eliminated double spotting from uronic acids. A butanol : formic acid : water mixture (500 : 115 : 385 V/V) equilibrated to contain butyl formate according to Edington and Percival (6) was used for O-methyl uronic acids.

Colors were developed on the dried papers by dipping them in 2.5 % aniline trichloracetate in glacial acetic acid and heating at about 100° C (10). In our hands the colors obtained with the several uronic acids vary. The basic color of all in visible light is a yellow-brown. The most reliable difference is the lack of fluorescence under ultraviolet from D-galacturonic and L-guluronic acids and its presence with D-glucuronic and D-mannuronic acids. In addition, with D-glucuronic and D-mannuronic acids, pink colors often, but not reliably, appeared on standing overnight at room temperature after heating. Such color never appeared with the other 2 uronic acids. L-Guluronic and D-mannuronic acids were obtained in a crude formic acid hydrolysate of alginic acid (17, 7). D-Mannuronic acid was also obtained as the lactone through the courtesy of the Kelco Corp., San Diego, California.

COLORIMETRY: Quantiative uronic acid analysis of the anion exchange resin eluates was performed in duplicate after treatment with acid washed charcoal (19) using the carbazole method (5) after McComb and McCready (14). The anthrone—$H_2SO_4$ test was used as adapted by Bailey (3) for the detection of pentoses and hexoses (0.0075 % anthrone) and with 0.1 % anthrone for hexoses alone (5).

## Experimental Results

IDENTIFICATION: When the original concentrated extracts containing some 35 to 55 % solids were spotted directly on the chromatographic papers, only the overripe apple and the ripe and overripe tomato samples showed any uronic acids. Both a yellow-brown and a pink spot appeared in the apple samples (see below). The acids isolated from all the fruit extracts contained only 1 uronic acid. This gave a color identical to that given by D-galacturonic acid. One overripe apple and 1 overripe tomato sample were examined by co-chromatography. The uronic acid was readily distinguished from all but D-galacturonic acid (table I).

TABLE I
SEPARATION OF URONIC ACIDS AND RELATED COMPOUNDS BY PAPER CHROMATOGRAPHY

| | Distance (cm) of spot center from origin | | $R_G$*** |
|---|---|---|---|
| | BPW* | BFW** | BFW** |
| D-Galacturonic acid | 6.6 | 3.5 | 0.03 |
| D-Glucuronic acid | 7.9 | 3.5 | |
| L-Guluronic acid | 7.6 | | |
| D-Mannuronic acid | 8.8 | 5.4 | 0.05 |
| Unknown uronic acid | 9.5 | 4.5 | |
| D-Mannuronolactone | | 10.7 | 0.13 |
| 2-O-methyl-D-galacturonic acid | | | 0.20 |
| D-Glucuronolactone | | 15.2 | 0.21 |
| 4-O-methyl-D-mannuronic acid | | | 0.25 |

* Butanol : pyridine : water (7 : 7 : 6 V/V) on pectate-treated Whatman no. 1; averaged values from co-chromatograms.

** Butanol : formic acid : water (500 : 115 : 385, V/V, equilibrated).

*** Ratio of distance of travel to that of tetramethyl glucose, from ref. (6).

A 2nd acid (in smaller amount) which yielded a red color immediately on heating (not just on subsequent standing, as with D-glucuronic and D-mannuronic acids) was found in all the apple extracts, but not the tomato extracts. The red color could be due to the presence of either a 2-O-methyl uronic acid (6) or a pentose containing acid. Examples of neither of these categories were available for comparison. However the unknown was easily distinguishable from compounds in the former category on the basis of their reported (6) chromatographic positions relative to the uronic lactones (table I). A small sample, separated from galacturonic acid by chromatography on thick paper (previously washed chromatographically with dilute HCl and water), gave a carbazole color reaction similar to galacturonic or glucuronic acids. In addition, the diluted carbazole and anthrone tests indicated the presence of significant amounts of both hexose and pentose.

TABLE II
URONIC ACIDS IN APPLES AND TOMATOES

| Fruit | Ripeness | Alcohol soluble uronic acid | Fruit firmness | Uronic acid of total pectin |
|---|---|---|---|---|
| | | *μg/g fresh fruit* | *lbs.* | *μg/g fresh fruit* |
| Stayman apple | unripe | 4 | 18 | 4390 |
| | ripe | ... | 16 | 4520 |
| | overripe | 470 | 8 | 3130 |
| Delicious apple | unripe | 12 | | 5500 |
| | ripe | 12 | | 4870 |
| | overripe | 390 | | 4310 |
| Valiant tomato | unripe | 14 | | 2140 |
| | ripe | 140 | | 2280 |
| | overripe | ... | | 2090 |
| Brookston tomato | unripe | ... | 23 | 2760 |
| | ripe | ... | 2 | 2880 |
| | overripe | 170 | 1 | 2800 |

QUANTITATIVE ANALYSIS: The results of the quantitative assays shown in table II confirm the gross differences in amounts of galacturonic acid and the above-mentioned unknown which were apparent on the paper chromatograms. For comparison, the uronic acid of the total fruit pectin was calculated from the carbazole assay on the isolated pectin (20) and shown in table II. Also shown are comparative figures on the firmness of the peeled fruit as determined with a Ballauf fruit pressure tester (20). The striking result obtained is that while uronic acid is present in all tested stages of these fruits, there is a very large increase upon softening of both species. Due to lack of some data for calculation, a figure for ripe Stayman apples is not shown, but it appeared quite low, as with Delicious apples. Only 3 tomato samples were analyzed. Quantitative data on the amount of the unknown uronic acid are not available, but as it was not present in as large amount as free galacturonic acid, the values in the table refer largely to free galacturonic acid.

## DISCUSSION

The amount of free uronic acid produced in the softened fruit is but a small fraction of the uronic acid present in a bound form as pectin. However, the decrease in the latter upon softening of the apples was only 1 to 3 times as great as the increase found in the alcohol fraction. The tomatoes show a different pattern, in that no fall in pectic uronic acid was found to accompany the increase of free uronic acid. Thus, the free uronic acid appears to be a more sensitive indicator of senescent changes than total pectin. Changes occurring within the total pectin fraction are discussed elsewhere (20).

Free uronic acids have been determined by others in several fruits recently. Using only a colorimetric method, Harris (9) and Winkler (18, 19) reported values in the range 10 to 50 $\mu$g/g for a number of apple varieties. Ash and Reynolds (2) could find none in ripe apples by their direct chromatographic procedure, which was not sensitive to such small amounts as we found. Borenstein et al (4) reported rather constant colorimetric values of 120 to 150 $\mu$g/g in ripe tomatoes during prolonged storage. These values agree with our results.

## SUMMARY

An acid fraction isolated from 70 % 2-propanol extracts of apples and tomatoes contained a component identical in $R_f$ and aniline color test to galacturonic acid. Guluronic, glucuronic, and mannuronic acids were not found. In addition, the apple samples contained a minor, but complex, uronic acid component. Data on the quantity of aqueous-alcohol-soluble uronic acid present at various stages of fruit ripeness showed very low values in unripe tomatoes and in ripe as well as unripe apples (4 to 15 $\mu$g/g fresh fruit). This increased at least 10-fold upon the softening of the fruit, but was still an order of magnitude less than the amount of D-galacturonic acid bound in pectin.

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# ENZYME ACTIVATION IN WHEAT GRAINS IN RELATION TO WATER CONTENT. GLUTAMIC ACID - ALANINE TRANSAMINASE, AND GLUTAMIC ACID DECARBOXYLASE [1, 2, 3]

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Information is extensive concerning seed germination, particularly physiology of seedlings (20). However, little is known concerning the physiological and related enzymatic activity of viable non-germinating seeds and grains at very early stages of germination or at moisture levels insufficient to produce germination. The latter condition is related to deterioration in storage.

The well-known increase in respiratory activity in dampened grains—in earliest studies attributed to physiological activity of the embryo (1, 2, 8)—has been related in the past decade primarily to the growth and metabolism of certain saprophytic fungi (3, 6, 13, 14, 15). More detailed study, particularly with wheat embryos and dicotyledonous seeds, has shown that a significant carbon dioxide release and a less pronounced oxygen uptake occur immediately upon wetting (5, 12). This effect is, however, transitory, followed a number of hours later by the strong respiration associated with the germination and growth of fungal spores. These results indicated that seed enzymes may cause this immediate gas exchange. The phenomenon was studied with excised wheat embryos (10). Also studied were changes in free amino and keto acids of intact wheat grains and excised embryos in relation to water content and germination (9). Results of these studies suggested that certain decarboxylases and transaminases are activated at lower moisture levels than are respiratory enzymes, and that the prior and selective dissipation of certain vital respiratory intermediates in storage may prejudice the subsequent germinative process. For these reasons the present investigation is primarily of relationship of water contents below the germination requirement to the glutamic acid decarboxylase and transaminase activities in wheat and excised wheat embryos.

[1] Received revised manuscript October 2, 1958.

[2] Contribution no. 301 of the Department of Flour and Feed Milling Industries, Kansas Agricultural Experiment Station, Manhattan, Kansas. This study was supported by a grant from the Rockefeller Foundation.

[3] Presented at the meeting of the American Society of Plant Physiologists, Bloomington, Indiana, August 25, 1958.

## MATERIALS AND METHODS

Fresh granular wheat germ containing only traces of endosperm material was obtained from International Milling Company, Minneapolis, Minn. Moisture content of the germ was 9.2 %. For experiments with grains, soft red winter wheat, variety Seneca, grown in Ohio, was used. Its moisture content was 11.6 %; total protein, 9.9 to 10.4 %; and germination above 97 %. Both materials were stored in sealed containers at +4° C.

Effects of various moisture levels in promoting enzymatic changes in amino and keto acids in wheat grains and excised germ were studied by wetting the ground material with and without added amino acid substrates. After the dampened tissue was incubated at 30° C, the amino acids were extracted and determined quantitatively by a two-dimensional paper chromatographic method (9). The detailed procedure follows:

Ten grams of wheat grains or 1 g of excised wheat germ was crushed in a small mortar in a calculated amount of 0.067 M. (M/15) phosphate buffer. Five mg of substrates (34 micromoles ($\mu$M) of glutamic acid, or 34 $\mu$M of $\alpha$-ketoglutaric acid and 56 $\mu$M of alanine) were dissolved previously in the buffer solution. The pH was adjusted to 5.8 for decarboxylase and to 7.3 for transaminase determinations. Reaction mixtures were incubated 3 hours at 30° C, after which the enzymes were inactivated with 70 % w/v ethanol. Amino compounds were extracted with 70 % w/v ethanol, purified with Amberlite IR-120 ($H^+$) cation exchange resin and concentrated in vacuo to 1 ml. From this final solution, normally 5 microlitres were pipetted on the origin of a chromatogram.

Whatman no. 4 filter paper was used without prewashing for chromatography. *n*-Butanol:acetic acid: water (63:10:27) and phenol (Merck, reagent grade) saturated with water were used as solvents. Colorimetric quantitative determinations of amino acids were done according to Fischer and Dörfel (4).

Manometric assays for decarboxylase activity were carried out in Warburg flasks as previously described (10).

## Results and Discussion

Decarboxylation: Dry wheat embryos were dampened with phosphate buffer solution alone to moisture values of 15, 18, 25 and 40 %, respectively, and incubated 3 hours. (fig 1). Glutamic acid rapidly decreased, at moisture values of 25 % and up. A corresponding increase in γ-amino butyric acid (γ-AB) appeared beyond 25 % moisture content. Thus, at 40 % moisture content in this time interval, glutamate concentration fell from 6 to 3 μM, and γ-AB rose from 1.8 to 6 μM/g dry weight. Alanine increased only slowly from 8 to 9 μM/g dry weight under these conditions. When glutamate is added to this system (fig 2) the production of γ-AB increased greatly at the 40 % moisture level to 28 μM. Furthermore, at the higher initial glutamate concentration this compound disappeared at moisture values as low as 15 %. Alanine was not affected by the increased concentration of glutamate added to the dampened embryo material.

The reaction whereby glutamate is transformed to γ-AB increases only slowly with moisture levels beyond 15 % with dampened ground wheat grains, which originally contain comparatively much less glutamate, γ-AB, and alanine, than embryo tissue (fig 3). With moisture increases beyond 18 % other reactions occur which produce a net increase in glutamate. Moreover, alanine increases relatively faster in the whole grain than in excised germ. Figure 4 shows the effect of adding glutamate to the whole grain-buffer mixtures at various moisture levels. Reactions that occurred then were analagous to those in germ to which glutamate had been added, i.e., a marked decrease in glutamate accompanied by an increase in γ-AB and a slower increase in alanine.

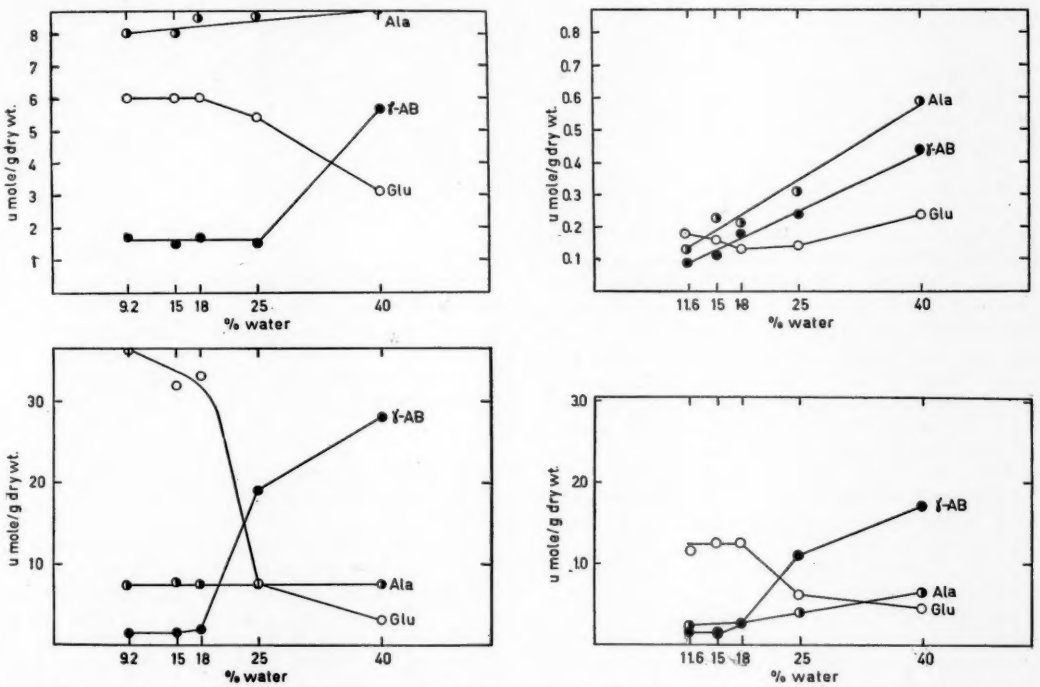


Fig. 1 (*top left*). Effect of dampening wheat embryos with phosphate buffer solutions on the concentration determined chromatographically of alanine (Ala), γ-aminobutyrate (γ-AB) and glutamate (Glu) originally present in the tissue, after 3 hours of incubation at 30° C.

Fig. 2 (*bottom left*). Effect of dampening wheat embryos with phosphate buffer solution containing added glutamate, on the concentration determined chromatographically of alanine (Ala). γ-aminobutyrate (γ-AB), and glutamate (Glu), after 3 hours of incubation at 30° C.

Fig. 3 (*top right*). Effect of dampening ground whole wheat grains with phosphate buffer solutions on the concentration determined chromatographically of alanine (Ala), γ-aminobutyrate (γ-AB), and glutamate (Glu) originally present in the tissue, after 3 hours of incubation at 30° C.

Fig. 4 (*bottom right*). Effect of dampening ground whole wheat grains with phosphate buffer solution containing added glutamate, on the concentration determined chromatographically of alanine (Ala), γ-aminobutyrate (γ-AB), and glutamate (Glu), after 3 hours of incubation at 30° C.

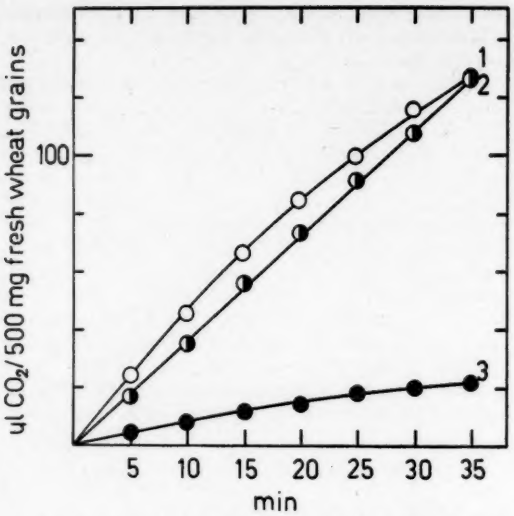


FIG. 5. Location of glutamic acid decarboxylase in wheat grains and in portions of wheat grains determined by manometric technique. 1) germ ends; 2) whole grains; 3) endosperm ends.

The destruction of glutamate observed is clearly a decarboxylation which results in the production of γ-aminobutyrate. A crude but active glutamic decarboxylase enzyme preparation was isolated from excised wheat embryos according to the procedure of Suzuki and Takakuwa (19). It may also be noted that decarboxylation of glutamate was immediately and completely inhibited by adding 70 % w/v ethanol, thus confirming the enzymatic nature of this reaction.

Results of applying manometric techniques to determine the site of glutamic acid decarboxylase in wheat are shown in figure 5. For this test embryo ends of wheat grains were carefully cut off with a razor blade so that no germ material remained in the endosperm part. Germ ends of wheat kernels as well as whole grains (1 and 2, respectively, fig 5) showed a high sustained rate of decarboxylation of glutamic acid, whereas the non-germ ends of the grains (3 fig 5) showed very low activity. The small contamination of the embryo material with starchy endosperm was of no importance in view of the small overall decarboxylative activity of the endosperm part.

TRANSAMINATION: The relation between water content and transamination in wheat grains was also studied. Schales and Schales (17) have shown recently that transamination of α-ketoglutarate (α-KGA) with aspartate or alanine is an important preliminary step in the decarboxylation of α-KGA. Linko and Milner suggested that these reactions form the initial step in the water-induced dissipation of α-KGA in grain during storage (9). To test this possibility, as shown in figure 6, alanine and α-KGA were added to the buffer solution used to dampen the wheat germ, which was then incubated for 3 hours at the various moisture levels shown. An activation of transaminase appeared, indicated by a rapid drop in alanine concentration and a corresponding increase in glutamate at moisture levels as low as 15 %, a value less than the moisture requirement previously shown for the decarboxylation of glutamate. A similar situation appeared in whole grains (fig 7), except that in the germ tissue the glutamate formed appeared to be more readily decarboxylated to γ-AB.

The manometric technique proved useful (fig 8) to demonstrate that wheat embryo tissue induced both aspartic acid and alanine to enter transamination reactions with α-KGA. Figure 8 also shows that no

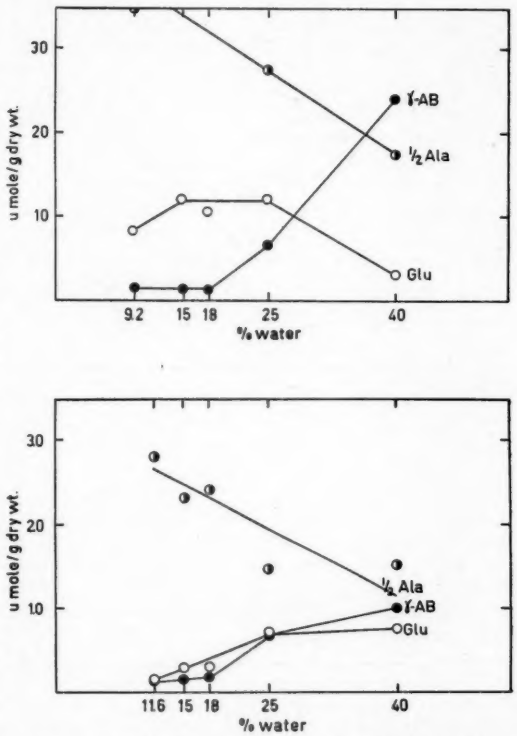


FIG. 6 (*top*). Effect of dampening wheat embryos with phosphate buffer solutions containing alanine (Ala) and α-ketoglutarate, on the concentration determined chromatographically of glutamate (Glu) and γ-aminobutyrate (γ-AB) after 3 hours of incubation at 30° C.

FIG. 7 (*bottom*). Effect of dampening ground whole wheat grains with phosphate buffer solution containing alanine and α-ketoglutarate, on the concentration determined chromatographically of glutamate (Glu), and γ-aminobutyrate (γ-AB) after 3 hours of incubation at 30° C.

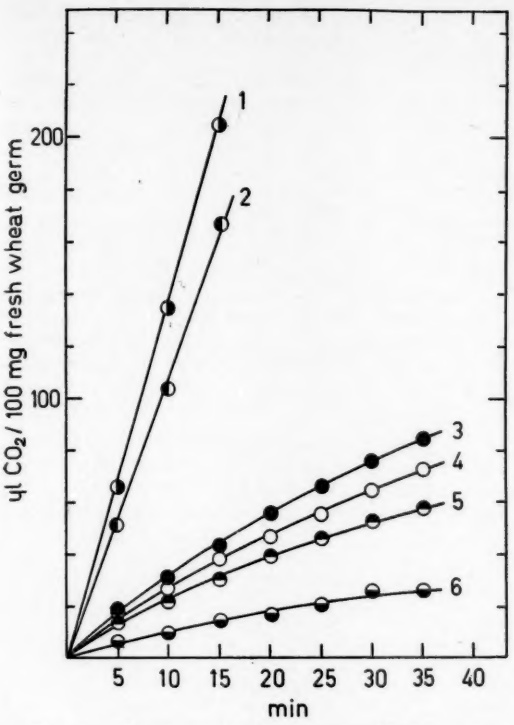


FIG. 8. Wheat germ-induced transamination of α-ketoglutarate with various compounds, using manometric technique. 1) α-ketoglutarate + alanine; 2) α-ketoglutarate + aspartate; 3) α-ketoglutarate; 4) α-ketaglutarate + glutamine; same curve for α-ketoglutarate + threonine; 5) α-ketoglutarate + α-aminobutyrate; 6) aspartate.

transamination with α-KGA that would cause additional decarboxylation of α-KGA occurred in the presence of glutamine, threonine or γ-aminobutyrate. This confirms results of Schales and Schales (17) based on other plant materials. Aspartic acid alone was decarboxylated by the embryo tissue but not nearly so readily as was α-KGA alone.

The effect of malonate on glutamic acid decarboxylation and alanine-glutamic acid transamination was investigated by chromatographic techniques (table I). Forty-eight micromoles (µM) of malonate per 1 g of tissue has a slightly inhibitory effect on both reactions in the intact germ.

The presence of glutamic acid decarboxylase in wheat grains has been shown previously (7, 16), but these authors did not consider the significance of water alone in the activation of the enzyme. Hence, the question remained whether any enzyme activity is present in the intact grains at normal storage moisture levels. This present study shows that glutamic acid decarboxylase activity is very slight in dry grains but that this enzyme is rapidly activated by adding water. The same appears to be true of glutamic acid – alanine transaminase. Results of these experiments, in addition to the earlier discoveries (9), indicate that water induced dissipation of α-KGA in grains is due to transamination of α-KGA to form glutamate, which then is decarboxylated. This is additional proof that the irreversible dissipation of such vital intermediates as α-KGA and glutamate, which is characterized by significant carbon dioxide evolution, may be a primary cause for loss of viability in stored seeds. That decarboxylation can occur in dry wheat embryos subjected only to elevation of atmospheric humidity has already been demonstrated (10).

Despite the presence of glutamic acid decarboxylase in wheat grains, no significant accumulation of γ-AB occurs during normal germination (9). This supports recent discoveries with isotopic tracer techniques, which have led to the conclusion that γ-AB is not merely an end product of a side reaction but rather an important metabolic intermediate in higher plants (11, 18).

## SUMMARY

Enzymatic decarboxylation and transamination of glutamic acid were studied in wheat grains and excised wheat embryos by chromatographic and manometric techniques, in relation to water content. Moisture levels as low as 18 % activate both enzyme systems whose activity increases rapidly with moisture content up to that required for germination of the grain. Transamination of α-ketoglutarate with alanine occurred at moisture levels as low as 15 %. At higher

TABLE I

EFFECT OF MALONATE ON GLUTAMIC ACID DECARBOXYLASE AND ALANINE–GLUTAMIC ACID TRANSAMINASE IN WHEAT GRAINS DURING THREE HOURS OF INCUBATION FOLLOWING WETTING *

| REACTION MIXTURE | ALA | GLU | γ-AB |
|---|---|---|---|
| 1. G (not wetted) | 2.3 | 4.2 | 3.1 |
| 2. G + B | 4.1 | 1.5 | 3.1 |
| 3. G + B + Glue | 4.8 | 15 | 11.4 |
| 4. G + B + Glu + Ma | 3.1 | 18 | 7.7 |
| 5. G + B + Glu + 70 % ethanol | 3.3 | 22 | 3.1 |
| 6. G + B + Glu | 4.1 | 11 | 9.7 |
| 7. G + B + Glu + Pyr | 7.2 | 11 | 6.8 |
| 8. G + B | 4.1 | 3.1 | 2.9 |
| 9. G + B + α-KGA | 2.3 | 3.8 | 4.0 |
| 10. G + B + α-KGA + Ala | 12 | 9.3 | 7.9 |
| 11. G + B + α-KGA + Ala + Ma | 12 | 7.7 | 6.2 |

* In terms of concentration of compounds involved, expressed as µM per g dry weight. In all cases moisture content was adjusted to 25 %. pH was adjusted to 5.8 in mixtures 1 through 5, and to 7.3 in mixtures 6 through 11. Following abbreviations are used: G = grains; B = buffer; Glu = glutamate; Ala = alanine; γ-AB = γ-aminobutyrate; α-KGA = α-ketoglutarate; Pyr = pyruvate and Ma = malonate.

levels, transamination is followed by rapid decarboxylation of glutamate. Glutamic acid decarboxylase is located almost entirely in the embryo. In the intact grains, other metabolic reactions overcome the decarboxylation of glutamate at moisture levels higher than 18 %, resulting in a net production of glutamate. Significance of these results is discussed.

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# METHODS FOR THE EXTRACTION OF ENZYMES FROM CEREAL LEAVES WITH ESPECIAL REFERENCE TO THE TRIOSEPHOSPHATE DEHYDROGENASES [1, 2]

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The authors have experienced considerable difficulty with the extraction and purification of the triosephosphate dehydrogenases from leaves of cereal plants during investigations of the respiratory and photosynthetic enzymes of leaves. Published methods (5, 9, 14) have proved unsuitable for the preparation of active enzyme extracts from immature and mature leaves of corn, wheat, oats, and barley.

This paper describes 3 methods for the extraction and purification of triosephosphate dehydrogenases from cereal leaves. Although these procedures were designed primarily for the extraction of triosephosphate dehydrogenases, they are also suitable for the extraction of many other enzymes. The preparative steps are simple and rapid and are recommended for the routine investigation of leaf enzymes, as well as for methods that are suitable for the classroom demonstration of the principles of enyzme extraction, purification and assay.

The procedures deal largely with the extraction of

[1] Received October 15, 1958.

[2] This investigation was supported by a National Science Foundation Grant no. 4407 and by Federal funds granted by the Hatch Act.

[3] This work was conducted in the Crop Physiology Laboratory of the Agronomy Department, University of Illinois and while E. R. Waygood was a visiting professor.

the enzymes from leaves of mature corn plants, but they have been tested and found to apply equally well to the extraction of enzymes from leaves of wheat, oat and barley plants.

## Methods and Materials

Immature or mature leaves of several varieties of corn as well as of samples of wheat, oats and barley were harvested from the University of Illinois Agronomy farm and transported to the laboratory in pails of tap water at the ambient temperature. Mid-ribs were removed from the corn leaves. The leaves were washed and the surface water was removed by blotting. The leaves were then cut into small pieces prior to grinding in a Waring blendor for 1 to 2 minutes. For maximum yields the final temperature of the homogenate should not exceed 4 to 8° C. Storage of the cut leaf material in the cold (−10° C) for 30 to 60 minutes prior to homogenization did not lower the level of enzymatic activity of the extracts.

Enzyme Assays: *Triosephosphate dehydrogenases*: The DPN[4] and TPN linked enzymes were assayed by a modification (8) of the spectrophotometric method (18). The concentration of enzyme in 3.0 ml of reaction mixture was 100 $\mu$g of protein or less depending on the state of purification. The substrate, 3-phosphoglyceraldehyde, was prepared (17) from the DL-glyceraldehyde-1-bromide-3-phosphoric acid dioxane addition compound obtained from Merck & Co., P.O. Box 899, Montreal, Canada. One micromole was added to 3 ml of reaction mixture.

An enzyme unit is defined as that amount of enzyme that catalyzes the reduction of pyridine nucleotide at the rate indicated by an increase in optical density ($D_{340}$) of 0.01 units per minute. Specific activity is expressed as enzyme units per mg protein.

Protein concentration of partially purified extracts was calculated by the method of Warburg and Christian (19), using the factor $D_{280}/D_{260}$ to correct for nucleic acids. It has been shown (9) that the results obtained with this protein procedure are comparable to those obtained by the method of Lowry et al (11), with partially purified plant extracts. The protein of crude extracts was determined by Nesslerization of 5 % trichloracetic acid precipitable material (16).

*Hill reaction*: The activity of illminated chloroplasts was measured qualitatively by the reduction of the dye, 2,6-dichlorophenolindophenol. Chloroplasts were prepared as described in Method II, step 1 (see below). One milliliter of the resuspended chloroplasts was diluted 10-fold with 0.05 M phosphate–0.01 M KCl (pH 7) buffer and 0.2 ml of a water solution of the dye (3.0 mg %) was added. This mixture was evenly divided into 2 test tubes, one of which was covered with aluminum foil to exclude light. The test tubes were then placed in a beaker of water at room temperature and illuminated by a 150-watt incandescent lamp placed 3 inches from the beaker for 3 minutes. Hill reaction activity is shown by decolorization of the dye in the illuminated tube. The dye has a maximum light absorption peak at 540 m$\mu$.

[4] The following abbreviations are used: DPN and TPN for di- and tri-phosphopyridine nucleotides respectively; TRIS for tris (hydroxymethyl) aminomethane and EDTA for ethylenediaminetetraacetic acid.

## Experimental Results

Methods previously reported for the extraction of DPN and TPN-triosephosphate dehydrogenases from sugar beet or pea leaves require the preparation of either a) acetone powders from the juice obtained from the leaves, (9, 14) or b) aqueous extracts obtained by grinding large amounts of pea leaves in a minimum amount of macerating medium (6, 7). Acetone powders made from juice obtained by grinding mature corn leaves in a Juicex extractor (obtained from Drachenberg Products Manufacturing Co. 23600 Harper Avenue, St. Clair Shores, Michigan) possessed a very low level of activity. It was difficult to grind the mature, fibrous corn leaves without heating or clogging the juicer. Mature corn leaves macerated in a mortar acording to the procedure of Gibbs (7) gave extracts which possessed low specific and total activity but were comparable to the pea leaf extracts. However, if the proportion of grinding medium to leaves was increased, the resulting extract had little or no activity. Corn, wheat or oat leaves extracted with phosphate or similar buffers (1:4 w/v (with or without EDTA)) by grinding in a mortar or Waring blendor, or powders prepared by grinding the tissue in cold (−20° C) acetone (1:10 w/v) were inactive. Carbowax 4000 extracts prepared according to the method of Clendenning et al (2) were also inactive. Carbowax is the trade name of a graded polymer of ethylene glycol obtained from Carbide and Carbon Chemical Co., 230 N. Michigan Ave., Chicago, Illinois. The extracts of cereal leaves prepared by these methods were not activated by the 10-minute preincubation with cysteine that was used in the routine assay.

However, the addition of cysteine to the macerating medium permitted the preparation of active acetone powders or extracts by any of these procedures. The triosephosphate dehydrogenases could then be purified from the extracts by a combination of Carbowax 4000 and acetone fractionation. Since fractionation with ammonium sulfate resulted in a rapid deterioration of the enzymes, its use was discontinued. One of the major difficulties encountered in the final stages of purification has been the removal of material that has a strong light absorbing capacity at 260 m$\mu$ and assumed to be nucleic acids.

Three methods for the preparation of solutions of triosephosphate dehydrogenases are described step by step to a stage of high purification.

Method I—Cysteine-phosphate Extraction: *Step* 1: One hundred grams of leaf tissue are ground in a cold Waring blendor for 1 to 2 minutes with 400 ml 0.01 M cysteine and 0.05 M phosphate buffer, pH 7.0, (with or without 0.005 M EDTA). The

brei is pressed through cheesecloth and centrifuged[5] for 20 minutes at 20,000 × G. The supernatant liquid is decanted and assayed.

*Step 2*: The supernatant liquid is adjusted to pH 7.0 and 40 g of Carbowax 4000 added per each 100 ml. The addition of the Carbowax increases the volume by 20 % which makes a final Carbowax concentration of 30 % and it also raises the pH to 7.5. The enzymes may be stored by freezing at this stage.

*Step 3*: The solution, after thawing if stored, is centrifuged for 10 to 20 minutes at 20,000 × G. The supernatant liquid is discarded and the precipitate is resuspended in 225 ml of phosphate-cysteine, pH 7.0 and centrifuged again for 10 minutes at 20,000 × G. Resuspension is facilitated by using a motor driven Teflon pestle directly in the centrifuge tube. Again, Carbowax is added at the rate of 40 g per 100 ml. This solution is centrifuged at 20,000 × G and the precipitate discarded. The discarded residue contains a higher proportion of DPN than TPN enzyme. The supernatant liquid is adjusted to pH 5.5 and the precipitate that forms is then collected by centrifugation and resuspended in 100 ml phosphate-cysteine, pH 7.0 and clarified by centrifugation if necessary.

*Step 4*: A half volume of cold (−20° C) acetone is added, slowly, with stirring to the final solution from step 3. The precipitate which forms is removed by centrifugation and discarded. Then, additional cold acetone is added to the supernatant liquid to 50 % concentration which precipitates the bulk of the triosephosphate dehydrogenases. This precipitate is collected by centrifugation, resuspended in 50 ml of cysteine-phosphate buffer, pH 7.0, and recentrifuged to clarify. The supernatant liquid is adjusted to pH 5.5 and Carbowax 4000 added (40 g/100 ml) and frozen. This material also has been stored at 2° C for a period of a week without an appreciable loss of activity. The acetone precipitates may also be removed by vacuum filtration using a Buchner funnel and Celite as a filter aid.

The data presented in table I are typical of the results obtained by processing 100 g of mature corn leaves according to Method I.

[5] All centrifugations are carried out in refrigerated centrifuges.

Some of the preparations were treated with activated charcoal (Darco G-60) and Alumina Cδ (obtained from Sigma Chem. Co., 3500 DeKalb Street, St. Louis, Mo.) after the acetone fractionation and yielded highly active preparations with some loss perhaps due to adsorption. Maximum specific activities obtained for these treatments were 2,800 for the DPN enzyme and 4,000 for the TPN enzyme, with protein concentrations of 0.08 mg/ml in a volume of 50 ml, thereby yielding 11,200 and 16,000 units.

METHOD II—CYSTEINE-CARBOWAX 4000 EXTRACTS: As indicated by the work of other investigators (2, 12, 15) the addition of Carbowax 4000 to the grinding medium of step 1 Method I permits the preparation of chloroplasts active in the Hill reaction. While this addition also eliminates a step from Method I in the extraction of the triosephosphate dehydrogenases it yields an initial extract of lower activity.

*Step 1*: One hundred grams of leaf tissue are ground in a Waring blendor for 2 minutes with 400 ml of a macerating medium prepared by adding Carbowax 4000 at the rate of 40 g to each 100 ml 0.05 M phosphate–0.01 M cysteine pH at 6.5. All other procedures are identical with the successive steps described in Method I. The specific activity at this stage is 40 for the DPN enzyme and 80 for the TPN enzyme with a protein concentration of 6.2 mg/ml, in a volume of 400 ml, thereby yielding between 100,000 and 200,000 units.

Chloroplasts for Hill reaction studies may be obtained by resuspending the precipitate from the 1st centrifugation in an equal volume of either 0.05 M phosphate–0.01 M KCl, pH, 7.0 (without cysteine) or this buffer mixture and Carbowax 4000 at a concentration of 30 % (40 g/100 ml).

METHOD III—ACETONE POWDERS: *Step 1*: Fifty grams of leaf tissue are ground in a Waring blendor for 2 minutes with 500 ml of cold (−20° C) acetone and 25 ml of a mixture (pH 8.0) of 0.2 M cysteine, 0.03 M EDTA and 0.1 M TRIS. A detailed description of the procedure is published (8). The yield of dry powder varies from 10 to 20 g per 100 g fresh leaves depending upon maturity.

*Step 2*: Twenty grams of the powder are extracted with 500 ml of cold (2° C) 0.05 M phosphate buffer and 0.005 M cysteine (pH 7.8). The slurry is stirred or mechanically agitated for 15 minutes in the

TABLE I

SUMMARY OF PURIFICATION PROCEDURE OF TRIOSEPHOSPHATE DEHYDROGENASE FROM 100 GRAMS OF MATURE CORN LEAF TISSUE

| STEP | VOLUME ML | PROTEIN MG/ML | SPECIFIC ACTIVITY DPN | SPECIFIC ACTIVITY TPN | TOTAL ACTIVITY DPN | TOTAL ACTIVITY TPN |
|---|---|---|---|---|---|---|
| Cysteine-phosphate extract | 400 | 5.8 | 86 | 110 | 200,000 | 255,000 |
| Carbowax suspension pH 7.5 before centrifugation | 510 | 4.9 | 70 | 97 | 175,000 | 242,000 |
| Carbowax fractionation | 200 | 2.2 | 173 | 395 | 76,000 | 174,000 |
| Acetone fractionation | 100 | 1.2 | 550 | 970 | 66,000 | 116,000 |

cold (2° C) and then centrifuged at 20,000 × G for 20 minutes. The residue is discarded. The best specific activities that were obtained with the supernatant liquid were 202 for the DPN enzyme and 412 for the TPN enzyme, with a protein concentration of 0.98 mg/ml and a volume of 425 ml, thereby yielding 84,000 and 172,000 units. The material may now be handled as described in Method I, step 2.

OTHER ENZYMATIC REACTIONS: Chloroplasts isolated from mature corn leaves by the procedure described in Method II were active in the Hill reaction. Qualitative measurements indicated that the illuminated chloroplasts decolorized from 50 to 75 % of the added dye, 2,6-dichlorophenolindophenol, in 3 minutes.

Cysteine-Carbowax extracts (Method II, step 1 and the successive purification step 3 of Method I) contained other enzymes that were assayed by standard methods (3). The following enzymes were detected in these extracts: catalase, carbonic anhydrase, enolase, glucose-6-phosphate dehydrogenase, isocitric dehydrogenase, malic dehydrogenase, "malic enzyme," nitrate reductase, oxalacetic carboxylase, and peroxidase. Enzymes that were not detected, due to their absence or inactivation during extraction were; glutamic acid dehydrogenase and decarboxylase, glutathione reductase, and glyoxylic acid reductase. It is possible that modifications in the extraction medium, time, temperature or pH would be required for maximum yield or detection of other enzymes, since the procedures were designed for the extraction of triosephosphate dehydrogenases.

The extracts from the acetone powders (Method III) contained aldolase and glucose-6-phosphate dehydrogenase activity in addition to the triosephosphate dehydrogenases. These extracts were not surveyed for other enzymes.

## DISCUSSION

Active preparations of the triosephosphate dehydrogenases can be made from immature or mature cereal leaves provided that cysteine is used in the grinding medium and all subsequent steps. Although cysteine has been used to activate the DPN and TPN triosephosphate dehydrogenases (4, 5), its use as a protectant during aqueous extraction or preparation of acetone powders has not been reported. A similar use of cysteine has been reported for the extraction and purification of carbonic anhydrase (20) and for nitrate reductase (13).

All of these enzymes have been associated with photosynthetic activity or found solely in photosynthetic tissue. The TPN dependent triosephosphate dehydrogenase has been found only in photosynthetic tissue (5) and its activity has been directly associated with light (9). It has been shown in this laboratory that the TPN linked enzyme is absent in etiolated corn leaves and is present in very low concentrations in albino plants. The specific activities of the DPN and TPN enzyme in these plants were as follows: etiolated, DPN = 99, TPN = 0.0; albino, DPN = 148, TPN = 21; green, DPN = 136, TPN = 132. Carbonic anhydrase also has been suggested as an enzyme concerned with photosynthesis and has been found to be localized in the cytoplasm of leaf tissue in plants (20). Candela et al (1) using cauliflower plants found a definite association of nitrate reductase with light.

The fact that all of these enzymes are protected by cysteine during extraction may have some significance in the physiology of leaves. The activity of the sulphydryl enzymes may depend on the production and maintenance of a high concentration of sulphydryl groups during photosynthetic activity. When these enzymes are disrupted and diluted during extraction, they may be rapidly oxidized unless protected.

That the inactivation of the triosephosphate dehydrogenases is not primarily due to heavy metals is indicated by the failure of EDTA (without cysteine) to preserve the activity in either the aqueous extracts or acetone powders. Assuming then that the inactivation of the enzyme is due to oxidation of the sulphydryl groups, it would appear that atmospheric oxygen is not the oxidant. This conclusion is based on the complete lack of activity of extracts prepared (without cysteine) in the Waring blendor in an atmosphere of nitrogen.

Other sulphydryl compounds will also protect the triosephosphate dehydrogenases during extraction. On comparable lots of mature corn leaves, glutathione proved superior (10 %) and sodium thioglycolate inferior (15 %) to cysteine as a protectant. The requirement for cysteine or similar sulphydryl compounds during extraction may vary with the sulphydryl content of the source material. Thus the requirement may vary not only between species but also upon the stage of development in a given species. Hopkins and Morgan (10) found that although the increase in sulphydryl groups with germination was ubiquitious, the content varied markedly with the different plant families. Members of the Gramineae were lowest in sulphydryl content.

Carbowax 4000 was first used by McClendon (12) for isolating active chloroplasts from red algae and leaves of higher plants. He observed precipitation of nucleoprotein during its use. Stocking (15) found that necleoprotein and amylophosphorylase were precipitated in Carbowax extracts of leaves. Clendenning et al (2) attribute the beneficial effect of Carbowax on the Hill reaction activity of chloroplasts partly to the precipitation of soluble protein which may be lost from the chloroplasts during the sucrose extraction procedure and partly to the removal of leaf tannins. These experiments indicate that Carbowax 4000 has considerable value as an enzyme precipitant especially in leaf breis in which many enzymes are unstable.

Method I has been found to be most reproducible, convenient and also gives the maximum yield of enzyme activity. The advantage of Method III is that it effects partial purification and thereby yields initial extracts of highest specific activity. It is not as reproducible as Methods I and II. This variability is

due, at least partially, to the age and type of material and the pH of the acetone slurry after grinding. Whereas it is unnecessary to add buffer to the acetone when young etiolated corn leaves are used as the source material, buffer at pH 7.8 to 8.2 must be added to obtain maximum activity from mature leaves. It is suggested for best results with all methods that an optimum pH and buffer concentration should be established for each particular source material.

## Summary

Methods were developed for the extraction and purification of enzymes, especially triosephosphate dehydrogenases, from immature or mature cereal leaves. Two facets of this work were; 1st, the addition of cysteine to the grinding medium for the preparation of active acetone powders or aqueous extracts, and; 2nd, the use of Carbowax 4000 as one of the enzyme precipitants. Acetone fractionation was used for the final stages of purification. These techniques permit the preparation of highly purified triosephosphate dehydrogenases in good yields from small (100 g) amounts of leaf tissue. Qualitative tests demonstrated that these methods are also suitable for the extraction of many other enzymes from cereal leaves.

Appreciation is expressed to Donna Flesher and Almut Gitter for technical assistance and to Dr. D. E. Alexander for the supply of albino corn seeds.

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# EFFECTS OF ARTIFICIAL INCREASES IN SUGAR CONTENT ON FROST HARDINESS[1]

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In an earlier paper (3), it was shown that infiltration of cabbage (*Brassica oleracea* L.) and *Saxifraga cordifolia* leaves with glycerine induced increases in frost hardiness. These increases were accounted for quantitatively on the basis of a purely osmotic effect. Since sugars commonly increase during hardening to frost, it is of fundamental importance to determine whether the same explanation can be applied to these substances.

## METHODS

Any increase in hardiness due to an osmotic change in the cell sap is proportional to the initial hardiness and would be immeasurably small in unhardened plants (2); therefore, as in the case of the earlier investigation with glycerine (3), hardened plants were used in the following experiments. Since plants are less permeable to sugar than to glycerine, greater difficulty was experienced in getting the sugars into the cells. The leaves of Saxifraga were nearly always injured during the evaporation of the intercellular water after infiltration with sugar. Consequently, all the investigations were confined to cabbage leaves. The procedure was as follows. One- to two-month-old cabbage seedlings were hardened at 3 to 5° C under artificial light (12 hrs per day) for 10 to 25 days. Two or three similar leaves, as determined by their numerical position above the lowest leaf, were removed from each plant and placed alternately in 2 piles, reversing the order each time. One pile of leaves was infiltrated in a vacuum desiccator with half molar sugar solution, the other pile with distilled water. In order to make sure that infiltration with water did not in itself alter the hardiness of the controls, in some cases the control leaves were not infiltrated; this did not alter the results. The leaves were then blotted and placed in covered Petri plates about 2 feet below a 200-watt bulb in the cold room. By keeping the leaves covered in this way, evaporation proceeded slowly enough to permit absorption of the sugars into the leaf cells without any osmotic damage. Evaporation of the excess water was complete in 24 hours. In order to obtain a sufficient increase in cell sap concentration, the infiltration was performed 3 times on 3 successive days. At the end of this time, the solution causing incipient plasmolysis in each set of leaves was determined, using $CaCl_2$ as plasmolyte and testing 3 leaves of each treatment. The concentration ratio (sugar infiltrated : control) was calculated from the conversion table (2) as in the case of the glycerine experiments. Frost hardiness was determined by suspending the leaves in a freezing chamber at $-2$ to $-3$° C, inoculating with snow to insure freezing, and lowering the temperature gradually. Two or three leaves were removed at each of a series of temperatures that covered a range of injury from 0 to 100 %. The temperature that produced an average injury of 50 % was considered the frost killing point. Zero injury occurred at 2 to 3° C above this point, 100 % injury at 2 to 3° C below it.

## RESULTS

Smaller increases in cell sap concentration were obtained with sugar than with glycerine. This difference was partly due to the lower permeability of the cells to the sugars, requiring the use of less concentrated solutions (half molar instead of molar), but also partly because the measured increase was less than expected from the amounts of sugar used. Thus, when infiltrated 3 times with $\frac{M}{2}$ sugar, the expected increase was 7 to 9 atms. The actual increase was only 3 to 4 atms. This was undoubtedly due to metabolism of a portion of the sugar after it entered the cell. Tests with $I_2$ in KI failed to reveal the formation of any starch as a result of infiltration, but there was a development of anthocyanin pigment. Evidence that metabolism was involved was obtained in later experiments, in which the light was filtered through a layer of water in an open, transparent, plastic tray. In this way, the leaves were maintained at a lower temperature than when exposed directly to the light, and no anthocyanin was formed. The increase in osmotic potential under these conditions was raised to 5 to 6 atms. (table I). At any one temperature, the difference between the injury to the

[1] Received October 16, 1958.

TABLE I

EFFECT ON HARDINESS OF INFILTRATION OF CABBAGE LEAVES WITH SUGARS

| STATE AND NO. OF LEAVES USED | INFILTRATION TREATMENT | OSMOTIC POTENTIAL (ATMS) | CONCENTRATION RATIO (SUGAR INFILTRATED : CONTROL) | FROST-KILLING POINT (° C) | |
|---|---|---|---|---|---|
| | | | | DETERMINED | CALCULATED |
| 10 to 14 day hardened (32 leaves) | $H_2O$ | 16.1 | | −7.5 | |
| | 0.5 M dextrose | 20.0 | 1.235 | −9.5 | −9 |
| 10 to 14 day hardened (36 leaves) | $H_2O$ | 15.0 | | −7.0 | |
| | 0.5 M fructose | 18.6 | 1.27 | −9.5 | −9 |
| 14 to 22 day hardened (54 leaves). Light filtered through water | $H_2O$ | 15.7 | | −6.5 | |
| | 0.5 M fructose | 21.3 | 1.36 | −9.0 | −9 |

controls and the sugar infiltrated leaves was found to be statistically significant (at the 5 % point if 8 to 10 replicate leaves were used; at the 1 % point if 12 to 15). Thus 15 control leaves frozen at −6.5 to −7° C showed an average of 52 % injury (±32 at the 1 % point), 15 fructose infiltrated leaves only 6 % (±10 at the 1 % point).

Table I shows that the increases obtained with dextrose and fructose agree with the calculated values, assuming a purely osmotic effect. In view of Jeremias' (1) pronounced increases in frost hardiness as a result of treatment with D-ribose, this sugar was also tried. As in the case of the hexoses, at any temperature resulting in partial frost injury, the average injury was less in the ribose infiltrated leaves than in the controls. Unlike the results with the hexoses, however, the differences were not statistically significant (though 3 experiments were averaged) and therefore are not presented here. The results do establish the fact that ribose does not confer any more frost resistance than do the hexoses.

## DISCUSSION

Since the differences between the control and the hexose-infiltrated leaves are statistically significant, there is no question that infiltration with hexoses does increase frost hardiness. Yet this increase is smaller than the normal increase during hardening (about 2° instead of 4° in cabbage) though the artificial increase in osmotic potential is larger (as high as 5 to 6 atms.) than the natural increase (about 3 atms.). Therefore, the increase in sugars can account for only part of the natural increase in hardiness and there must be some other hardiness factor.

It may be objected that these results apply only to the luxury zone—i.e., to hardy plants that already possess enough sugar for marked hardiness. But this objection is invalid, for it has already been shown by previous investigators (2) that if non-hardy plants with low sugar content undergo either a natural or an artificial increase in sugar content, this fails to induce an appreciable increase in hardiness. This applies both to non-hardy plants that are capable of hardening and those that are not. It, therefore, seems safe to conclude that in both non-hardy and hardy plants, an increase in sugar content increases hardiness simply due to the osmotic properties or to some other colligative properties of the cell sap. Since this effect is proportional to the hardiness in the absence of the sugars, it is negligible in the non-hardy plants, but measurable and equal to the calculated value in hardy plants.

Since ribose failed to increase hardiness any more than did the hexoses, Jeremias' (1) results could not be confirmed. He reports greater frost resistance in wheat seedlings as a result of soaking the seeds in D-ribose than when he soaked them in dextrose. It has already been suggested (4) that his measurement of frost resistance is unreliable, since he froze for too short a time to obtain temperature equilibrium (20 to 60 min). Whether this or some other reason explains his results only further experiments can decide.

## SUMMARY

1. Infiltration of frost-hardened cabbage leaves with dextrose or fructose resulted in an increased frost hardiness equal to the calculated increase on the basis of a purely osmotic effect.

2. Infiltration with D-ribose failed to produce any greater increase than infiltration with either of the hexoses.

This investigation was supported by National Science Foundation Grant 4427.

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# DISTRIBUTION AND PROPERTIES OF ISOCITRITASE IN PLANTS[1]

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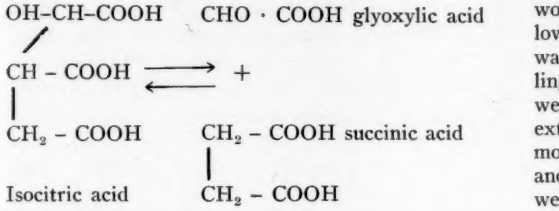
Isocitritase or isocitric lyase is the enzyme which catalyzes the reversible cleavage of isocitric acid to succinic and glyoxylic acids.




It was described originally in extracts from species of Pseudomonas and has since been shown to be present in a variety of bacteria (2, 9, 11, 12, 13) and fungi (8, 11, 13).

The metabolic significance of isocitritase appears to be that it catalyzes one of the steps of the glyoxylate cycle (8), which provides a mechanism by which acetate units can be quantitatively converted to malate. Since malate may be regarded as a precursor of hexose, the reactions of this cycle appeared to offer a solution to the long standing difficulty of explaining the formation of carbohydrate from acetate units in fatty seedlings (6, 7). The germinating castor bean, a prime example of such a seedling contains the enzymes of the glyoxylate cycle (6, 7). In particular, isocitritase was shown present in amounts adequate to account for the rates of reaction observed in vivo.

Since this was the first report of the occurrence of isocitritase in cells other than those of fungi and bacteria, it was of interest to find whether the enzyme was of general distribution in higher plants and to inquire into its properties more closely. One intriguing possibility, denied in the outcome, was that the activity of this enzyme and an ancillary glyoxylic reductase might account for the widespread occurrence of glycolate in plant cells. In fact, isocitritase appears to be confined, rather strictly, to those tissues in which fat is being converted to carbohydrate. A preliminary report of this work has appeared previously (3).

## MATERIALS AND METHODS

PLANT MATERIALS: Castor beans, variety Cimarron, supplied by the Baker Seed Corporation, Vernon, Texas, were the principle source of isocitritase preparations. The castor beans were soaked for 24 hours in water with a sprinkling of Arasan (disinfectant). The seeds were then transferred into vermiculite in wooden flats, thoroughly soaked with water and allowed to germinate at 30° C in an incubator. No water was added subsequently. Five-day-old seedlings, i.e., 5 days from placing the seeds in vermiculite, were used for the most part in the preparation of the extracts. The seedling proper and testa were removed, and the "beans," consisting of the endosperm and the enclosed cotyledons, were then washed and a weighed sample taken.

Other seeds examined for isocitritase activity were grown in a similar manner, except that the initial soaking was for a period of from 3 to 6 hours. The other plant materials mentioned were collected from the greenhouse or from commercial sources.

PREPARATION OF EXTRACTS: The plant material was ground in a blendor for 25 to 30 seconds with about 1.4 times its weight of 0.05 M potassium phosphate buffer, pH 7.6. The suspension so obtained was squeezed through muslin and centrifuged for 15 minutes at 12,000 × G in an International refrigerated centrifuge set at −7° C. All glassware and the buffer were previously cooled to 0° C. The top fatty layer which separated during centrifuging was discarded, and the supernatant solution, which usually contained from 15 to 22 mg protein/ml was used as the crude enzyme preparation.

PREPARATION OF SUBSTRATES AND OTHER CHEMICALS: *Isocitrate*: The monopotassium salt of D-isocitric acid lactone was a gift from Dr. H. B. Vickery and prepared for use according to his directions. Twenty-one and two tenths milligrams (100 $\mu$M) of the acid were dissolved in 1.8 ml of 0.1 *N* KOH and 1 ml of $H_2O$ and the solution warmed on a steam bath for 15 minutes. This was then adjusted to the desired pH (usually 6.0) with concentrated HCl and made up to 5 ml to give a concentration of 20 $\mu$M/ml. DL-Isocitric acid lactone, *allo*-free, from Sigma Chemical Company, St. Louis, Missouri, was prepared in a similar manner. Three ml of 0.1 *N* KOH was used to dissolve 17.6 mg (100 $\mu$M) of the acid lactone and the same procedure followed as with the D-isocitric acid. A fresh solution of isocitrate was prepared daily.

*Glyoxylate*: The monohydrate of sodium glyoxylate was obtained from Mann Chemical Laboratories, New York, N. Y. The dinitrophenyl hydrazone derivative was prepared and used as a standard for spectrophotometric and chromatographic identification of products of the isocitritase reaction.

[1] Received October 21, 1958.

[2] Present address: Monsanto Chemical Company, St. Louis, Missouri.

METHODS OF ASSAY FOR ISOCITRITASE ACTIVITY: Two methods were employed; each involved the estimation of the glyoxylate produced in the reaction mixture. In the 1st of these, aliquots were removed and analyzed by the Friedemann and Haugen method (4).

In a typical experiment, 200 $\mu$M (micromoles) of potassium phosphate buffer, pH 7.6, 15 $\mu$M of $MgSO_4$, 6 $\mu$M of cysteine HCl or glutathione adjusted to pH 7.6, 24 $\mu$M of isocitrate and water were added to a test tube to a final volume of 2.8 ml. At time zero, 0.2 ml of the enzyme preparation was added. One milliliter aliquots were taken at appropriate intervals and placed in a centrifuge tube containing 0.2 ml of 100 % trichloroacetic acid. After spinning down the precipitated protein, the supernatant was decanted into a test tube, and 0.33 ml of 0.1 % dinitrophenyl hydrazine in 2 *N* HCl added. After incubation for 15 minutes on a 30° C water bath, 1.67 ml of 2.5 *N* NaOH was added slowly. The solution (3 ml) was placed in a Corex cell and readings made at 445 m$\mu$ on a Beckman B spectrophotometer and compared with a glyoxylate standard.

The 2nd assay method was that used by Olson (personal communication), in which the reaction was followed continuously. Glyoxylate reacts with semicarbazide in the assay mixture and the production of semicarbazone is measured at 252 m$\mu$ on the spectrophotometer. The formation of the glyoxylate semicarbazone is catalyzed by acid and therefore the reaction must be carried out at pH 6.0 or less. (As will be shown later, this is not the optimum pH for the enzyme activity.) The reaction mixture consisted of 200 $\mu$M of potassium phosphate buffer, pH 6.0, 15 $\mu$M of $MgSO_4$, 6 $\mu$M of cysteine HCl or glutathione, and 60 $\mu$M of semicarbazide HCl adjusted to pH 6.0 with 0.1 KOH. Freshly prepared reaction mixture was placed in a cuvette with $H_2O$, and 0.05 ml of the enzyme preparation. At time zero, 0.3 ml of isocitrate (6 $\mu$M) was added to give a final volume of 3 ml. Generally in the enzyme experiments a linear rate of glyoxylate formation was attained only after a lag of 2 to 4 minutes. Enzyme activity was expressed as increase in optical density per minute over the linear portion of the curve, usually for a period of 4 to 6 minutes (fig 1). (O.D. at 252 m$\mu$/3.6 = $\mu$M glyoxylate). The progress of glyoxylate formation at 2 levels of isocitritase is shown in figure 1.

PROTEIN AND NUCLEIC ACID: Protein estimation and the determination of "percent nucleic acid," in the enzyme preparations were carried out by methods described by Layne (10).

## RESULTS AND DISCUSSION

ISOCITRITASE ACTIVITY OF PLANT EXTRACTS: In the earlier reports (6, 7) it was shown that, since the crude preparations may contain isocitric dehydrogenase, glyoxylic reductase and a common pyridine nucleotide, some of the added isocitrate might be converted to $\alpha$-ketoglutarate rather than to glyoxylate. No such interference was evident after ammonium sulfate precipitation, and completely unequivocal demonstration of isocitritase activity and the stoichiometry of the reaction were possible with such preparations (7). In the present survey, interference from isocitric dehydrogenase was prevented by first treating the crude preparations with about 5 mg Darco charcoal per ml. This treatment was shown to remove added triphosphopyridine nucleotide from crude extracts while the isocitritase was unaffected.

Table I shows the results of isocitritase assays on extracts of the 25 different plant materials which were tested. Those extracts in which only a trace or no activity was present are listed first (1 through 16) and the rest in order of increasing isocitritase content (table I).

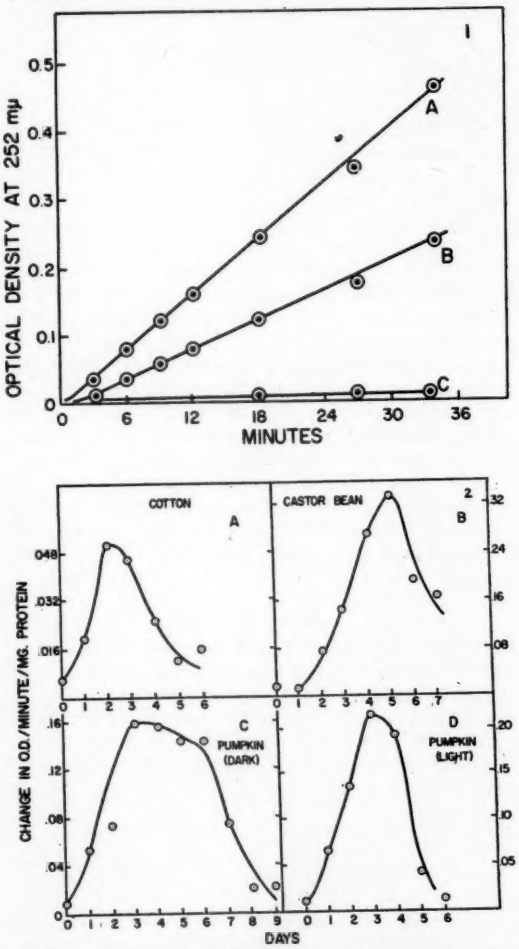



FIG. 1. Progress of the isocitritase reaction as measured by the semicarbazone assay. Cell A contained 0.10 ml isocitritase; cell B, 0.05 ml; and cell C, 0.10 ml boiled enzyme under the standard assay conditions.

FIG. 2. Changes in isocitritase activity during germination of various seeds.

The results show at once that isocitritase is not of universal distribution in plants. On the contrary, it is clear that it is confined to those tissues, principally fatty seedlings, in which an active fat metabolism may be anticipated. Isocitritase was not detectable in roots and leaves and stems in which the predominant catabolic event is the breakdown of carbohydrate. Moreover it is noteworthy that in the castor bean seedling itself, which is the best source we have found, the enzyme is localized in the endosperm tissue, to which the fat is also confined, and it is not found in the adjacent parts of the seedling proper. Again, the enzyme was not found in castor bean seeds maturing on the parent plant.

DEVELOPMENTAL STUDIES: (*a*) *In the germinating castor bean*: The endosperm tissue of the germinating castor bean contains virtually all of the food reserve of the seed. On the 3rd or 4th day of germination a rapid breakdown of the fat begins and by the 10th day the endosperm has been completely absorbed by the seedling proper. The disappearance of the fat is accompanied by the appearance of an almost equivalent amount of carbohydrate in the endosperm and in the young seedling (15).

It will be recalled that in the endosperm of the castor bean maturing on the parent plant, isocitritase activity could not be detected, while the 5-day-old seedling gave an extract with the highest specific activity of any tissue tested. The questions of the time sequence of development of the activity of the isocitritase, and of what happens to the activity as the endosperm is absorbed and disappears, thus became of considerable interest (figs 1 & 2).

TABLE I

ISOCITRITASE ACTIVITY IN VARIOUS PLANT TISSUES *

| | SOURCE OF PREPARATION | ACTIVITY * |
|---|---|---|
| 1. | Maturing castor bean endosperm | None |
| 2. | Castor bean root, 5-day-old seedling | " |
| 3. | Castor bean hypocotyl, 5-day-old seedling | " |
| 4. | Castor bean leaves | Trace |
| 5. | Apple fruit | None |
| 6. | Apple leaves | " |
| 7. | Barley seedlings, 3-day-old | " |
| 8. | Bean seedlings, 4-day-old | " |
| 9. | Carrot root | " |
| 10. | Coconut | " |
| 11. | Corn seedlings, 3-day-old | Trace |
| 12. | Pea seedlings, 4-day-old | Trace |
| 13. | Pepper fruits | None |
| 14. | Tobacco leaves | None |
| 15. | Tomato fruits | None |
| 16. | Oat seedling, 3-day-old | Trace |
| 17. | Sunflower cotyledons, 4-day-old seedlings | 0.004 |
| 18. | Flax seedlings | 0.022 |
| 19. | Soybean cotyledons, 3-day-old seedlings | 0.035 |
| 20. | Peanut cotyledons, 7-day-old seedlings | 0.047 |
| 21. | Avocado fruit | 0.048 |
| 22. | Watermelon cotyledons, 3-day-old seedlings | 0.074 |
| 23. | Cotton cotyledons, 3-day-old seedlings | 0.086 |
| 24. | Pumpkin cotyledons, 4-day-old seedlings | 0.216 |
| 25. | Castor bean endosperm, 5-day-old seedlings | 0.380 |

* Expressed as change in optical density at 252 mμ per minute per mg protein in the semicarbazone assay. (Charcoal treated preparations.) No activity is defined as a change in optical density of less than 0.001 per minute in the reaction mixture. Trace activity is defined as a change in optical density between 0.001 and 0.002 per minute.

Enzyme preparations were made at daily intervals and assayed for isocitritase by the semicarbazone method. Figure 2 B shows the isocitritase activity in germinating castor beans over a 7-day period. The activity after 24 hours of soaking was very low and it did not increase during the next 24 hours. Beginning on the 2nd day, there was a striking increase in activity up to a peak at the 5th day. After this there was an equally striking decline. It is therefore clear that during the 1st few days of germination isocitritase is produced or becomes active in the endosperm. What is more significant perhaps is that the enzyme activity reaches its peak at the time of most active fat breakdown in the seed. The later decline coincides with the softening and absorption of the endosperm material into the seedling proper. (*b*) *In germinating pumpkin and cotton seeds*: In the germination of the castor bean, the endosperm is, by the 5th day, in a state of decreasing metabolic activity and it is, perhaps, not unexpected that isocitritase activity should also decline. On the other hand, in seeds such as those of pumpkin and cotton, the fat is stored in the cotyledons, and the tissue remains metabolically active long after the fat has disappeared. It was of interest, therefore, to compare the isocitritase activity of the 2 different types of fatty seeds during germination.

Isocitritase activity in the germinating cotton seed is shown in figure 2 A. Here again there is an initial striking increase in activity, and a rapid decline occurs thereafter. By the 6th day, activity was only 20 % of that of the peak on the 2nd day, in spite of the fact that the cotyledons were still actively expanding.

Heydeman (5) recently reported that in marrow, a variety of *Cucurbita pepo*, isocitritase activity increased up to at least 6 days. In a study with pumpkin, another variety of *Cucurbita pepo* (fig 2 C), it was found that, in the dark, isocitritase activity increased until the 3rd day, then leveled off to about the 6th day, after which there was a rapid decline. When pumpkin seedlings were grown in the greenhouse exposed to sunlight, isocitritase activity once again reached a peak at the 3rd day (fig 2 D) and isocitritase activity declined even more rapidly than in seedlings grown in the dark.

From the 4 developmental studies carried out, it appears that isocitritase activity in the germinating seedling is ephemeral whether the fat is stored in the endosperm, which is completely absorbed during germination, or whether it is contained in cotyledons which persist as functional leaves. Enzyme activity, which was very low in the ungerminated seeds, rose to a peak when fat breakdown was at its height, and then declined again.

The picture which emerges from the results in this section is that the isocitritase enzyme is confined in the castor bean plant to the germinating seedling

and still further localized in the endosperm tissue. The likelihood that isocitritase is intimately concerned with the fat to carbohydrate conversion in the various seedlings is still further enhanced by the striking increases which occur in its activity as the seedlings progress from the dormant condition to the time of rapid fat mobilization, and by the disappearance of the enzyme as the seedling becomes independent of its fatty reserves. Such changes in enzyme activity are not, of course confined to isocitritase; our previous work has shown that mitochondrial activities show somewhat similar changes.

The appearance and disappearance of the isocitritase during germination can be compared with the behavior of the enzyme in microorganisms. When the cells are grown on carbohydrate as a carbon source, the enzyme is absent, but it appears, apparently "adaptively" when the cells are made to grow on acetate as the sole carbon source (11, 13).

PURIFICATION OF ISOCITRITASE: As shown in the preceding section extracts of 5-day-old castor bean seedlings had a higher specific isocitritase activity than any other source examined. In addition to this qualification as a source of isocitritase activity, the castor beans germinated well, were resistant to infection, and were uniform in growth.

The following purification procedure was adopted after many empirical trials, the details of which will not be reported here. In most of the experiments, from 150 to 200 g of endosperm were used, in 50 to 55 g lots, and the volume of the crude enzyme preparation (see Materials and Methods) was approximately 1 ml per 1 g of endosperm tissue used. This preparation usually contained from 15 to 22 mg of protein per ml. Solid ammonium sulfate was added to the crude preparation to a concentration of 22 %. This was done while the preparation was immersed in an ice bath and stirred by a magnetic stirrer. The preparation was allowed to stand at 5° C for 1 hour, then centrifuged for 15 minutes at 12,000 × G using the refrigerated centrifuge. The supernatant was then made to an ammonium sulfate concentration of 45 % as described above, and again kept at 5° C for 1 hour. The precipitated protein which contained from 70 to 80 % of the total enzyme activity was centrifuged and the supernatant discarded. Three-fourths of the original volume of 0.05 M potassium phosphate buffer, pH 7.6, was added and the protein was allowed to go into solution gradually with occasional swirling while at 0 to 5° C. $Ca_3(PO_4)_2$ gel was added in the ratio of 15 mg of gel to 100 mg of protein, and allowed to stand for 10 to 15 minutes before centrifuging. The precipitate was discarded. The $Ca_3(PO_4)_2$ gel step was repeated on the supernatant using the same ratio of gel to protein. (The use of larger ratios of $Ca_3(PO_4)_2$ resulted in loss of isocitritase activity.) The solution, which was now straw-colored and clear, was made up to 20 % with respect to $(NH_4)_2SO_4$, and then to 32 % in the manner described earlier; and the final precipitate was taken up in 0.05 M buffer. Two further $Ca_3(PO_4)_2$ gel treatments as described above usually resulted in a 9- to 14-fold purification. A final ammonium sulfate fractionation of 25 to 31 % provided a clear solution containing only minute amounts of nucleic acid, and 30 to 45 % of the total original isocitritase activity, with a specific activity of 18 to 30 times that of the original extract (table II). Such enzyme preparations were inactivated by dialyzing against either buffer, or buffer with cysteine and $MgCl_2$, and were used in the investigation of properties without further treatment.

TABLE II

ISOCITRITASE PURIFICATION PROCEDURE

| STEPS IN PURIFICATION | ACTIVITY * | PROTEIN MG/ML | NUCLEIC ACID | ACTIVITY / MG PROTEIN |
|---|---|---|---|---|
| Crude preparation | .100 | .327 | 12 % | 0.306 |
| 22 to 45 % $(NH_4)_2SO_4$ | .115 | .193 | 10 % | 0.600 |
| $Ca_3(PO_4)_2$ Gel 15:100 to protein ** | .113 | .118 | 7 % | 1.000 |
| $Ca_3(PO_4)_2$ Gel 15:100 to protein | .113 | .093 | 9 % | 1.200 |
| 20 to 32 % $(NH_4)_2SO_4$ | .131 | .063 | 4 % | 1.570 |
| $Ca_3(PO_4)_2$ Gel 15:100 to protein | .130 | .044 | 2 % | 2.900 |
| $Ca_3(PO_4)_2$ Gel 15:100 to protein | .131 | .036 | 2 % | 3.640 |
| 25 to 31 % $(NH_4)_2SO_4$ | .150 | .016 | 1 % | 9.400 |

* Semicarbazone assay.

** After each treatment with $Ca_3(PO_4)_2$, the material precipitating with the gel was discarded; the supernatant solution was used for the succeeding step.

PROPERTIES OF ISOCITRITASE: (*a*) *Sulfhydryl requirement*: Sulfhydryl and divalent cation requirements for isocitritase activity have been reported by several workers (11, 12, 14).

As shown in figure 3, cysteine and gluthathione were equally effective as activators of the castor bean enzyme over the concentration range tested. Increase in enzyme activity was linear with increase in sulfhydryl concentration from 0 to 1 $\mu$M/ml, with no further increases in activity at higher levels. By extrapolating the curve to the abscissa, it can be deduced that the residual sulfhydryl concentration in the enzyme preparation was about 0.1 $\mu$M/ml.

The minimum concentration necessary to give maximum activity of the enzyme (1 $\mu$M/ml) agrees closely with that reported by Smith and Gunsalus (14) in a recent description of the properties of a bacterial isocitritase. However, whereas these authors found that cysteine was more effective than gluthathione, the 2 were equally effective as activators of the castor bean enzyme.

(*b*) *Divalent ion requirement*: Seven divalent ions were tested; $Mg^{++}$, $Mn^{++}$, $Co^{++}$, $Fe^{++}$, $Cu^{++}$, $Ca^{++}$, and $Ni^{++}$. Only the 1st 4 caused an increase in isocitritase activity over the residual

TABLE III

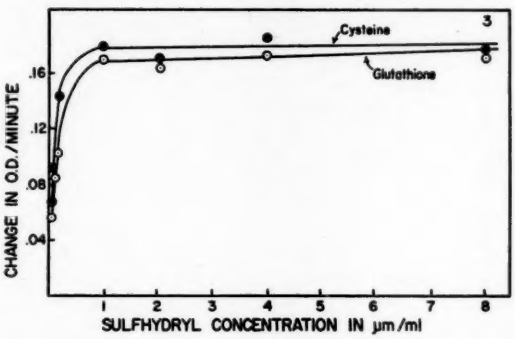
EFFECT OF pH ON ISOCITRITASE ACTIVITY

| pH | 5.5 | 6.0 | 6.5 | 7.0 | 7.5 | 8.0 | 8.5 |
|---|---|---|---|---|---|---|---|
| Activity as % of that at pH 7.5 | 31 | 50 | 58 | 52 | 100 | 87 | 66 |

value. Salts of these ions were then added at various concentrations to reaction mixtures. (The $Fe^{++}$ experiments were carried out by the Friedemann Haugen method because precipitation occurred when the carbazone mixture was used.) The results are shown in figure 4.

$Mg^{++}$ was superior to all other ions tested; a plateau was reached at a concentration of 1.5 $\mu$M/ml. $Mn^{++}$ and $Co^{++}$ were approximately 30 % as effective as $Mg^{++}$ at its optimum concentration in restoring isocitritase activity, and $Fe^{++}$ was somewhat more effective.

(*c*) *Influence of pH on the reaction*: Measurements of isocitritase activity by the Friedemann and Haugen assay at several pH levels between pH 5.5 and 7.5 are shown in table III. They show that the optimum for activity was pH 7.5. At pH 6.0, that used in the carbazone assay method, activity is only one half of that at pH 7.5 (table III).



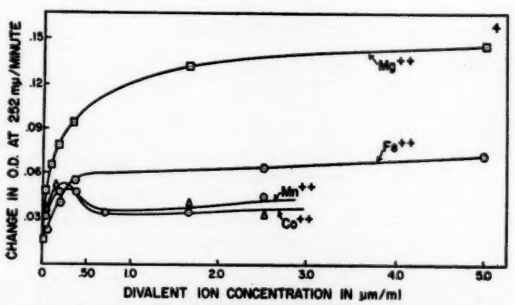
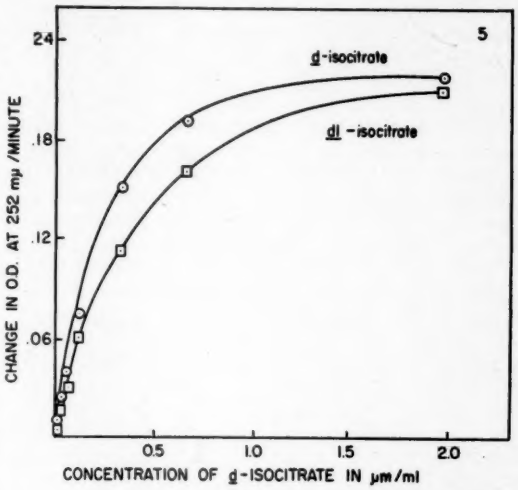




FIG. 3. The sulfhydryl requirement for isocitritase activity. The standard semicarbazone assay mixture was used, with sulfhydryl additions as shown.

FIG. 4. The divalent cation requirement for isocitritase activity. The standard semicarbazone assay mixture was used with the cations added as shown.

(*d*) *Sensitivity to heat*: The enzyme was rapidly inactivated at slightly elevated temperatures. Thus more than 50 % of the original activity was lost during 1 minute of exposure at 50° C and a 5 minute treatment at 40° C was sufficient to reduce activity by a similar amount.

(*e*) *Effect of substrate concentration*: The influence of isocitrate concentration on the rate of reaction is shown in figure 5. Rates obtained with



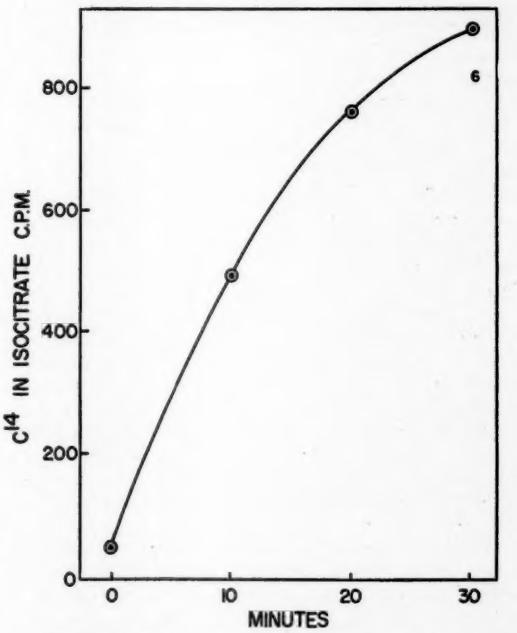




FIG. 5. Isocitritase activity as a function of substrate concentration.

FIG. 6. Progress of incorporation of succinate 2,3-$C^{14}$ into isocitrate. (See text.)

both the natural form and the DL mixture are shown, and these are plotted as a function of the D component only. It will be seen that at the lower levels, the rates of reaction in the presence of the L form are definitely lower than in its absence, while at 2 $\mu$M/ml the rates are virtually the same (figs 5 & 6).

The maximum rate of reaction is reached at a substrate concentration somewhat above 1.0 $\mu$M/ml. When the reciprocals of reaction rate and substrate concentrations were plotted against each other, a straight line was obtained and the Km was calculated to be $3.2 \times 10^{-4}$ M. The enzyme from *Pseudomonas aeruginosa* which was described recently had a Km of $4.5 \times 10^{-4}$ M (14).

(*f*) *Reversal of the reaction*: Although the function of isocitritase would appear to be the splitting of isocitrate, the reverse reaction can be shown to occur. For example Smith and Gunsalus (14) measured isocitrate synthesis by the enzyme from *P. aeruginosa*, although they emphasized that cleavage is strongly favored in dilute solutions.

The synthesis of isocitrate from succinate and glyoxylate by the castor bean enzyme was demonstrated in the following manner.

Twenty micromoles of succinate 2,3-$C^{14}$, 20 $\mu$M of glyoxylate, 10 $\mu$M $MgSO_4$ and 200 $\mu$M potassium phosphate, pH 7.5 were made up to a volume of 2 ml. At zero time 1 ml of an isocitritase preparation (18-fold purification) was added. Aliquots of 0.5 ml were removed at 0, 10, 20 and 30 minutes and placed immediately in a boiling water bath. They were then chromatographed on Whatman no. 1 paper using mesityl oxide:formic acid:water (200:41:200, v/v/v) as solvent. Radioautographs were subsequently prepared.

The only radioactive component other than residual succinate was one which moved very slowly indeed ($R_f$ 0.05) and which corresponded in position to known isocitrate. Figure 6 shows the progress of incorporation of radioactivity into this area during the experiment; after 30 minutes it was calculated that at least 8 % of the succinate had been so incorporated. The identity of this material with isocitrate was confirmed by the following observations.

1) Radioactive material eluted from the paper was co-chromatographed in another solvent (equal parts of propionic acid:water (62:79 v/v) and *n*-butanol:water (30:20 v/v) with known isocitrate, and the 2 were found to coincide at $R_f$ 0.25.

2) When the eluted material was incubated with fresh isocitritase under the conditions of the usual enzyme assay there was a rapid production of glyoxylate semicarbazone as judged spectrophotometrically.

3) After incubation with isocitritase the products were separated in the propionic acid:butanol solvent. A radioautograph revealed that the radioactive material at $R_f$ 0.25 had virtually disappeared and that 94 % of the radioactivity originally in the presumed isocitrate had now appeared in succinate.

The synthesis of isocitrate from succinate and glyoxylate under the conditions described above was found to be inhibited by malonate. Thus when malonate was included at a concentration equal to that of succinate, the incorporation of $C^{14}$ into isocitrate was reduced by 10 %; when the malonate:succinate ratio was increased to 5:1 the inhibition was 94 %.

## Summary and Conclusions

On the basis of the evidence from 25 different tissues it is concluded that, far from being widespread in higher plants, isocitritase is limited to certain species and confined, in these, to those parts in which active fat breakdown is occurring.

The developmental studies on germinating fatty seedlings showed that the early increases in enzyme activity and the later declines coincided with changes in the pace of fat breakdown. These findings furnish further presumptive evidence for the thesis advanced earlier (6, 7) that isocitritase activity is intimately concerned with the conversion of fats to carbohydrates in these tissues.

The isocitritase from castor beans has been somewhat purified. The best preparations had an activity some 30 times greater than that of the original extracts. The specific activity of such preparations is at least equal to that of the purified enzyme described recently from *P. aeruginosa*. The castor bean enzyme has several features in common with its counterpart from microorganisms. Thus a sulfhydryl compound and magnesium are mandatory for optimal activity which is shown at pH 7.5. The Km was found to be $3.2 \times 10^{-4}$ M. The enzyme was very sensitive to heat. The enzyme was shown to bring about the synthesis of isocitrate from glyoxylate and succinate, and malonate inhibited this reaction.

We are indebted to Dr. H. B. Vickery for a generous gift of D-isocitrate and to Dr. J. A. Olson for advance information about his isocitritase assay. W. D. Carpenter was an X–R Fellow at Purdue University and this paper is based on his Ph.D. thesis. We gratefully acknowledge the support of a grant from the National Science Foundation (G-5072).

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# CHANGES IN ENDOGENOUS GROWTH SUBSTANCES DURING FLOWER DEVELOPMENT [1]

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The theories advanced to explain the flowering phenomenon generally involve the assumption that specific substances are manufactured by plants at the time of flower initiation. Such are, for example, the views of Cajlachjan (1) who suggested the existence of a "florigen," of Melchers (15) who thought that a different substance, "vernalin," was involved in the flowering of biennials, of Hamner and Bonner (8), of Resende (25) who speculated on the possible role of native auxins and antiauxins, of Lang et al (14) who actually caused flowering in Hyoscyamus and Samolus with extracts of Echinocystis endosperm, etc.

Nevertheless, investigations on the natural regulators actually present in plants during the onset of flowering are rare and, in general deceiving. Thus, Cooke (3) found that the auxin level in Xanthium was related to day-length rather than to flowering, flowering plants placed under long days having higher auxin contents than similar plants placed under short days. On the contrary, Vlitos and Meudt (26) detected more auxin under short than under long days in 2 other short-day plants, soybean and the Maryland Mammoth tobacco. In long-day plants, Konishi (13) did not find drastic differences between the auxin levels of rosetted and bolting plants, despite the dramatic morphological difference between these 2 states. Finally, Gilson (6) concluded from his auxin determinations in induced and non-induced Hyoscyamus that the observed auxin fluctuations were a consequence rather than a cause of the flowering process.

The reason why the results of the cited experiments are often unconvincing is probably 2-fold: 1) the techniques used were crude and unspecific, and 2) no convenient and quantitative test for flower-forming substances has yet been devised.

In the light of this situation, it was attempted to take advantage of the more accurate methods known today for the determination of auxins and gibberellins in order to get a clearer picture of the fluctuations of these substances during flower development, even though these substances may have only an accessory role in the mechanism of flowering. Such a study is reported below. It has been presented as a Master's thesis (9) and short summaries of it have been published (11, 18).

## MATERIALS AND METHODS

PLANT MATERIAL: Three different physiological types of plants were used in the present study, namely: a long-day plant, *Rudbeckia speciosa* Wenderoth; a cold-requiring plant, *Chrysanthemum morifolium* Ram. variety Shuokan (cuttings were kindly supplied by Dr. H. M. Cathey, U.S.D.A., Beltsville, Maryland); and a short-day plant, *Chrysanthemum morifolium* variety Shasta. Rudbeckia appeared to be a favorable material because of its clear-cut response to photoperiodic treatments and also because its photoperiodic behavior has been studied in detail (5, 7, 13, 16). The Shuokan chrysanthemum is a Japanese variety (large pompon type, yellow) which bolts and flowers rapidly after a low-temperature treatment (23, 24). Unlike other chrysanthemums, it initiates and develops flowers regardless of the day-length. If the plant has not been exposed to cold, it remains vegetative and in a rosetted state for a long time[3]. The chrysanthemum Shasta is a short-day, commercial variety (anemone type, white) which usually requires 10 weeks to full bloom from the beginning of the short-day treatment.

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[3] At a minimum temperature of 16° C, bolting and flowering finally occur without cold treatment, but only after some 30 weeks, as was observed during the course of these experiments.

GROWING CONDITIONS: All plants were grown in a greenhouse which was maintained at a temperature of at least 20° C during the day and at 16° C during the night. Short-day treatments were given by means of covering the plants with black satin cloth from 5 P.M. to 8 A.M. every day. Long days were produced by lengthening the natural day to 18 hours with 60-watt incandescent light bulbs installed 5 feet apart and 2 to 3 feet above the plants.

The Rudbeckia seedlings were grown under short days until they reached a suitable size. The long-day treatments were started on Dec. 16, 1957. From that date on, 1 lot of 10 plants was moved to the long-day condition every week until the 1st lot had received 10 weeks of long days, at which time all the plants were harvested.

Cuttings of Shuokan chrysanthemums were taken from devernalized stock plants. Fifty uniform plants were selected, and were divided into 5 treatments (10 plants per treatment) as follows: 1 week, 2 weeks, 3 weeks, and 4 weeks of exposure to 1° C during the night, plus a control receiving no cold treatment at all. The cold treatments were given by moving the plants into a cold storage room (1° C) from 5 P.M. to 8 A.M. every day during the required period. During the day, the plants were kept in a greenhouse at about 20° C. The cold treatments for the 4 lots were started at the same time. Samples were taken immediately after the cold treatments.

The cuttings of Shasta chrysanthemums were placed under long days. A few days after potting, the terminal tips were removed, and 3 or 4 strong lateral shoots per plant were allowed to develop. The short-day treatments were started on Dec. 16; from this date on, 1 lot of 14 plants was brought into the short-day condition every week. After 10 weeks, samples were taken from all lots at the same time. The control lot consisted of plants which had been kept under long days during the entire period.

EXTRACTION OF THE ACTIVE SUBSTANCES: At the end of the various treatments the tips of the plants were harvested. Only the actual shoot apices with the young leaves enclosing them were used as material for extraction. The samples were brought immediately into a deep-freeze unit and stored at −23° C until lyophilized. Prior to lyophilization the frozen samples were crushed into small pieces in the deep-freeze with chilled mortar and pestle. The completely dried material was ground to a fine powder, and stored dry in a dessicator at 1 to 5° C.

Throughout this series of experiments, a uniform method of extraction was employed in order to obtain comparable pictures of the active substances occurring in the plant tissues. To identify the actual growth substances, or to purify the crude extracts, several other methods were tried. Cold methanol was used as the main extracting solvent as recommended by Nitsch (17). Unless mentioned otherwise, 50 mg (dry weight) of plant material were used for each extraction in the case of Rudbeckia and Shasta, and 25 mg in the case of the Shuokan chrysanthemum. The extraction was repeated 3 times using 20 ml of methanol each time. During the extracting process, the flasks containing the samples plus the extracting solvents were kept in an ice-bath in the refrigerator. The extracting solvent was changed every 20 minutes, so that the total extracting time for each sample was 1 hour. The methanol extract was evaporated to dryness under reduced pressure, the flasks being placed in lukewarm water. In general, the crude methanol extracts were used directly for chromatography.

CHROMATOGRAPHY AND BIOLOGICAL ASSAYS: Paper chromatography was used to separate the various active substances present in the extracts. The procedures and the techniques of ascending paper chromatography were based on the method described by Nitsch and Nitsch (20), using paper strips of Whatman 3 MM, 2 to 3 cm wide. Generally, isopropanol : water (80 : 20, v/v) was the developing solvent, though various other solvents were employed occasionally. The solvent was allowed to ascend 20 cm beyond the original spot in the dark.

The Avena 1st-internode (mesocotyl) test (21) was used in the present study to detect both auxins and gibberellins. The chromatograms were cut transversally into twenty 1-cm pieces, each of which was placed in a small tube with 10 1st-internode sections and 1 ml of the testing solution (citrate-phosphate buffer pH 5.0, about $10^{-2}$ M; Tween 80, 0.1 %; sucrose, 2 g/100 ml). The tubes were rotated in the dark for 20 hours at 25° C on a roller-tube apparatus (1 rpm). After this time, the length of the mesocotyl was measured to the nearest 0.1 mm using a binocular with an ocular micrometer.

Since the Avena 1st-internode test reacts to both auxins and gibberellins (21, 22), an "Avena leaf test" was used to distinguish between these 2 types of substances. This leaf test was performed as follows: Brighton oat seeds were soaked in water for 2 hours, then laid down on several layers of wet tissue paper disposed at the bottom of a transparent plastic box. The box with the seeds was exposed to fluorescent red light (filtered through red cellophane) for 3 hours on the 1st day and for 1 hour each on the 2nd and 3rd days, in order to prevent the growth of the 1st-internodes. Altogether, the seedlings were grown for 72 hours in a darkroom maintained at 25° C with about 85 % relative humidity. The part of the seedling used for this test was a 4-mm section cut 4-mm above the 1st node. This section contained a segment of both the coleoptile and the enclosed 1st leaf. Measurements of the elongation of the 1st leaf were made after 48 hours. All other procedures and techniques employed in this test were the same as the ones used in the 1st-internode test, except that no presoaking was required and that distilled water was used instead of the buffer-sucrose mixture. The elongation of the leaf sections was found to be promoted by gibberellin $A_1$ and gibberellic acid (down to a concentration of $10^{-9}$ M), but not by indole-3-acetic acid. In solutions containing 1, 10 and 100 $\mu$g/l of indole-3-

acetic acid (IAA) and 0.1, 1, 10 and 100 μg/l of gibberellic acid (GA) were prepared as controls in the 1st-internode and the 1st-leaf tests, respectively.

## Results

A Long-day Plant, *Rudbeckia speciosa*: *Active substances*: Figure 1 gives the histograms obtained with cold methanol extracts of Rudbeckia grown under short days and under 1 to 5 weeks of long days. In these histograms any elongation which is equal or superior to that given by 1 μg/l of IAA is statistically highly significant.

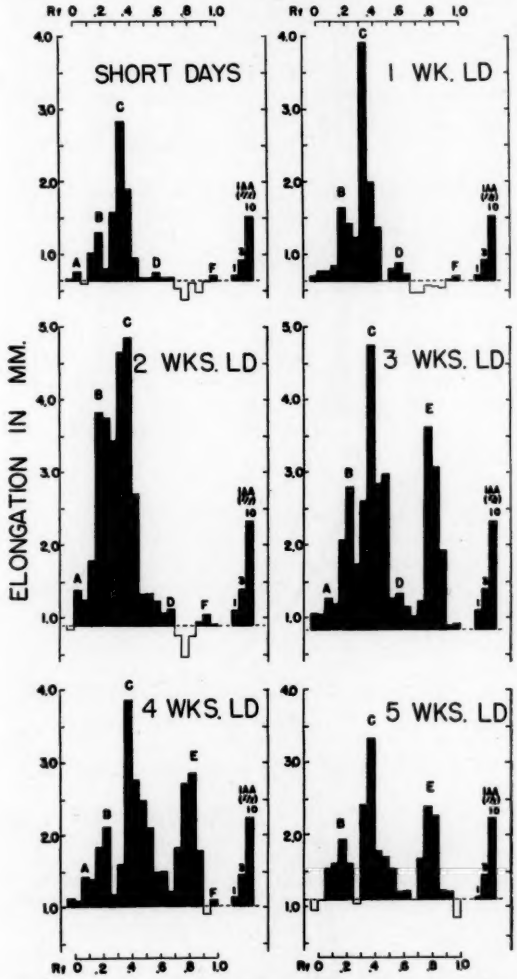


Fig. 1. *Rudbeckia speciosa*. Histograms representing the growth-promoting effect of 1-cm sections cut from paper strips on which the cold methanol extracts of 50 mg (dry weight) of stem tips have been chromatographed in 80 % isopropanol. Ordinates; average elongation of 10 oat 1st-internodes in 24 hours above the initial 4-mm length. Abscissa; $R_f$. The elongation produced by 1 ml of standard solutions containing 1, 3, 10 μg/l of IAA is indicated on the right of each histogram.

As can be seen on the histograms, at least 6 substances promoting the growth of Avena 1st-internodes over the controls in buffer plus sucrose can be distinguished in the crude, cold methanol extracts of the Rudbeckia tips. These substances have been called provisionally $A_1$, $B_1$, etc, and have the following $R_f$ values in 80 % isopropanol: $A_1$ (0.0 to 0.1), $B_1$ (0.1 to 0.25), $C_1$ (0.25 to 0.45), $D_1$ (0.5 to 0.65), $E_1$ (0.65 to 0.85), and $F_1$ (0.9 to 1.0). Substances $A_1$, $D_1$ and $F_1$ appeared either to have a low growth-promoting activity or to be present in very small amounts. On the other hand, substances $B_1$, $C_1$ and $E_1$ were major growth substances which promoted a remarkable elongation of the 1st internodes. Substance $E_1$, for example, extracted from only 50 mg of dried material caused in 20 hours a 3-fold increase in length of the 1st-internode sections.

Preliminary investigations on the nature and properties of substances $B_1$, $C_1$ and $E_1$ have yielded the following results:

Substance $B_1$ remains in the aqueous phase when shaken with either ether or chloroform at pH 2.8 to 3.0. It is not tryptophane (which has an $R_f$ of 0.3 in 70 % isopropanol, whereas the $R_f$ of $B_1$ is 0.1 to 0.2 in that solvent). It is active on both the 1st-internode test and the Avena leaf test.

Substance $C_1$ moves to the IAA position in 80 % isopropanol, but is not IAA because it also remains in the aqueous phase when partitioned between water and chloroform or ether at pH 2.8 to 3.0.

Substance $E_1$ is neither the nitrile nor the ethyl ester of IAA for its $R_f$ is 0.0 in petroleum ether (boiling point 30 to 60° C) : chloroform : $H_2O$ (75 : 15 : 10 v/v), whereas that of IAN is 0.40 and that of IAE is 0.80 in the same solvent. It is not gibberellin $A_1$ nor gibberellic acid either (which have an $R_f$ of 0.45 in 80 % isopropanol), although it is active on both the 1st-internode and the Avena leaf tests.

*Changes occurring during flowering*: When grown under short days, *Rudbeckia speciosa* remains a rosette of leaves without bolting or flowering. At that time, substances $B_1$ and $C_1$ are the main growth substances which can be seen on the chromatograms (fig 1).

If the plants are moved to long days of 18 hours, then a physiological process is triggered off which results in bolting and flowering. After 1 week of long days, however, no change can be noticed in the growth rate of the stem of the plant, although the color of the leaves has become a lighter green and their position has changed from horizontal to erect. Yet, the chromatograms of the growth substances extracted from the stem tips already show a 10-fold increase in the level of substance $C_1$ (fig 2). There is still little change in the growth rate after the 2nd week of long days, but at that time the concentration of substance $B_1$ also increases to about 10 times the concentration present in the controls remaining under short days (fig 2). The 1st signs of bolting

can be seen around the 3rd week of long-day treatment. At this very moment, a new substance appears on the chromatograms, substance $E_1$ (fig 1 and 2). From that time on, until anthesis, this remarkable substance has been found in all the extracts prepared from the stem tips. As shown in figure 2, there are 2 periods during which stem elongation is rapid, 1 corresponds to bolting, the other to the growth of the pedicel of the inflorescence. Both periods are preceded by an increase in the levels of substances $B_1$ and $E_1$.

A COLD-REQUIRING PLANT, THE SHUOKAN CHRYSANTHEMUM: *Active substances*: In the methanol extracts of the stem tips of Shuokan chrysanthemums, several substances promoting the elongation of oat 1st-internodes could be separated and were tentatively named $A_2$, $B_2$, etc. Among these substances, $B_2$, $C_2$ and $E_2$ were most prominent (fig 3). It should be noted that the $R_f$ values of these substances in 80 % isopropanol ($B_2$ (0.1 to 0.25), $C_2$ (0.25 to 0.4), $E_2$ (0.65 to 0.75)) were practically identical with the $R_f$ values of the corresponding substances extracted from Rudbeckia. In addition to this similarity, a partitioning experiment indicated that substance $B_2$ remained in the aqueous phase when partitioned between water and ether at pH 2.8, as did substance $B_1$ of Rudbeckia.

*Changes occurring during the cold treatment*:

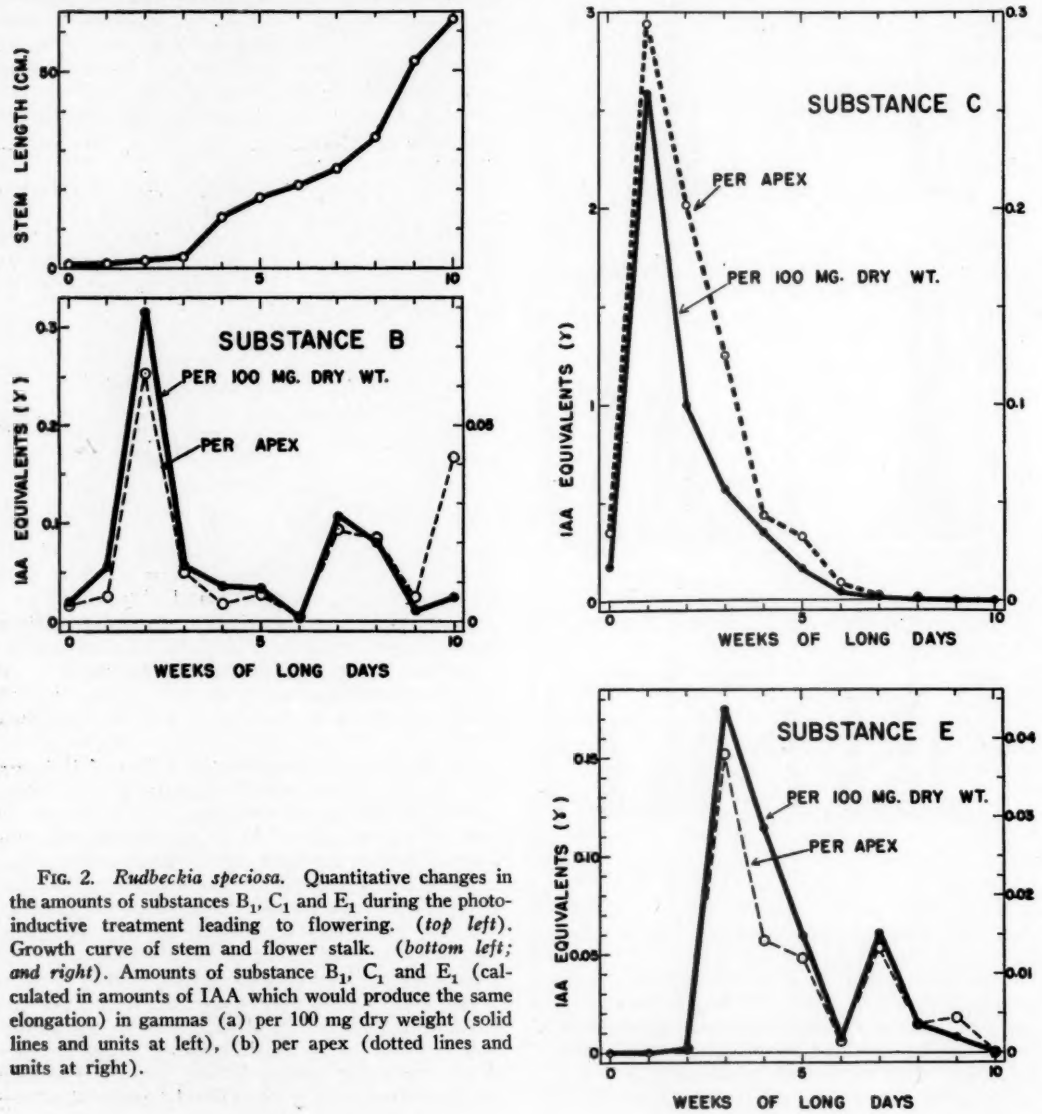


FIG. 2. *Rudbeckia speciosa*. Quantitative changes in the amounts of substances $B_1$, $C_1$ and $E_1$ during the photoinductive treatment leading to flowering. (*top left*). Growth curve of stem and flower stalk. (*bottom left; and right*). Amounts of substance $B_1$, $C_1$ and $E_1$ (calculated in amounts of IAA which would produce the same elongation) in gammas (a) per 100 mg dry weight (solid lines and units at left), (b) per apex (dotted lines and units at right).

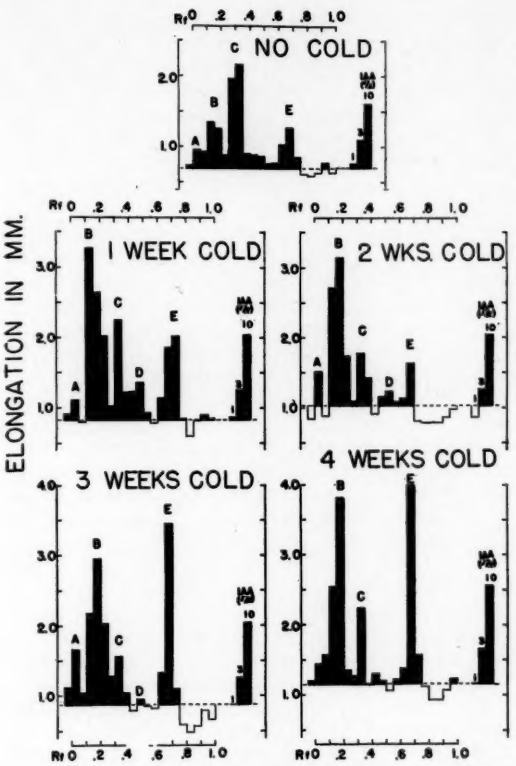


FIG. 3. Chrysanthemum variety Shuokan. Histograms corresponding to the cold methanol extracts of 25 mg (dry weight) of stem tips chromatographed in 80 % isopropanol. Ordinates and abscissa as in figure 1.

The Shuokan chrysanthemums were grown above a 16° C minimum temperature. Under these conditions they remained vegetative and rosetted for a very long time. When extracted in this state, the stem tips were found to contain relatively small amounts of growth substances, the main substance being substance $C_2$. Low amounts of substances $B_2$ and $E_2$ were also present (fig 3). During the vernalization process, the level of substance $C_2$ decreased, but that of $B_2$ increased, the concentration of $B_2$ rising to 10-fold its original level after 1 week of cold treatment (fig 4). On the 3rd week of cold treatment, substance $E_2$ suddenly rose to 10 times its concentration in the unvernalized controls. The timing of this change in the concentration of substance $E_2$ is worth noting, for it coincides with the minimum length of time which is necessary to vernalize the Shuokan chrysanthemum.

A SHORT-DAY PLANT, THE SHASTA CHRYSANTHEMUM: *Active substances*: As shown in figure 5, several growth substances were detected in the extracts of the tips of Shasta chrysanthemums. They had the following $R_f$ values: $A_3$ (0.0 to 0.1), $B_3$ (0.1 to 0.3), $C_3$ (0.3 to 0.4), $D_3$ (0.45 to 0.65) and, perhaps, $F_3$ (0.9 to 1.0). A toxic substance (which practically killed the 1st-internode sections) moved to the region between $R_f$ 0.6 and 0.9 (in 80 % isopropanol) and prevented the detection of growth-promoting substances in that area. Several techniques were tried in order to remove this inhibitor from the other substances, partitioning between water and ether at various pH values, selective adsorbtion on alumina columns and stepwise extraction of the lyophilized samples. It was found that the toxic substance was a neutral compound which could be extracted with ether from an aqueous solution at pH 8.0. None of the techniques tried, however, gave very satisfactory results, for the pattern of the growth substances changed after purification of the methanolic extract, indicating possible decomposition of the extracted substances.

In view of these results, the various samples of Shasta chrysanthemum were analyzed using the crude methanol extracts, as had been done with Rudbeckia and the Shuokan chrysanthemum.

*Changes occurring during flowering*: As shown in figure 5, the inhibitor was present at all stages of development, masking the possible presence of substances of the E type which would chromatograph at the same $R_f$. However, the changes in 2 prominent growth substances which were clearly separated from the inhibitor, namely $B_3$ and $C_3$ could be followed. Until the 5th week of short days, the amount of substance $C_3$ was greater than that of $B_3$. From the 6th week on, the reverse was true. This change coincided with the rapid development of the flower bud.

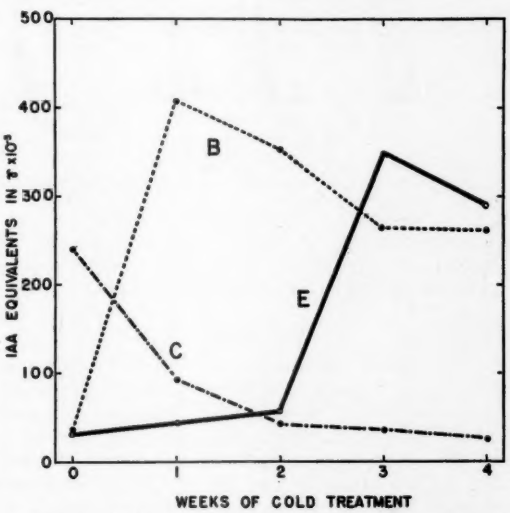


FIG. 4. Chrysanthemum variety Shuokan. Quantitative changes in substances $B_2$, $C_2$ and $E_2$ in stem tips during vernalization. Results expressed in amounts of IAA producing equal elongation of 1st internodes. Values given either per 100 mg dry weight or per apex (each apex happening to weigh about 100 mg dry weight).

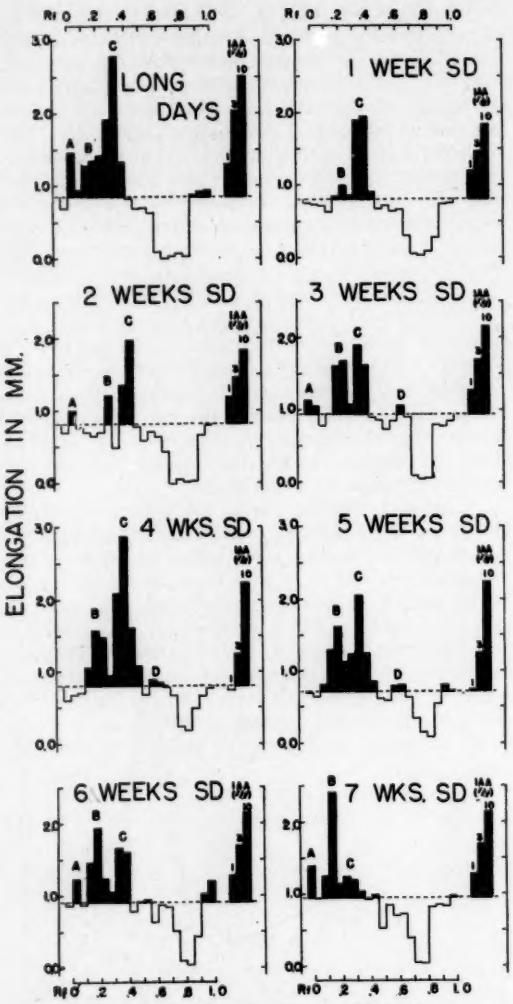


FIG. 5. Shasta chrysanthemum. Histograms corresponding to the cold methanol extracts of 50 mg (dry weight) of stem tips chromatographed in 80% isopropanol. Ordinates and abscissa as in figure 1.

## DISCUSSION

The results which have been presented above contribute experimental evidence to the idea that profound hormonal changes occur during flower development. These changes are both of quantitative and qualitative nature. As soon as the inductive treatment is started, the relative importance of the existing growth substances changes. When a plant becomes ready to bolt and flower, at least in the long-day and cold-requiring species studied, a new substance appears in the methanolic extract of the apices. It is to be remarked that the sudden increase in substances $E_1$ and $E_2$ precedes the actual bolting phenomenon by about 1 week in Rudbeckia and 2 weeks in the Shuokan chrysanthemum. It is more likely, therefore, that substance E is a cause rather than a consequence of the bolting phenomenon. Another observation seems to confirm the correlation between the presence of substance E and bolting; unvernalized Shuokan chrysanthemums eventually will bolt and flower after a very long time when grown above 16° C; this is a species in which low amounts of substance E exist even before vernalization.

Is substance E a florigen? This intriguing question must await further experimentation to be answered. The crucial point would be to know if substance E occurs in short-day plants once they are induced to flower. Unfortunately, the presence of a substance E in the Shasta chrysanthemum was completely masked by the toxic substance present in the methanolic extracts. At the present time it seems more likely that substance E may be a vernalin for it resembles the substance moving at $R_f$ 0.70 to 0.85 in isopropanol : ammonia : water (80 : 10 : 10, v/v) detected by Fukui et al (4) in vernalized lettuce seed.

In the preceding sentences, "substance E" has been spoken of as if substances $E_1$ and $E_2$ were identical. This point has not been proven as yet. However, there seems to be a resemblance between the pattern found in Rudbeckia and that found in the Shuokan chrysanthemum. In both cases, the main growth substances move to similar positions on the chromatograms and their quantitative changes follow the same pattern. The substances C (after an initial peak produced by long days in Rudbeckia) decrease steadily during flower development, the substances B increase then decrease, and substances E appear just before bolting. These resemblances between successive waves of endogenous growth regulators in a long-day and in a cold-requiring plant are reminescent of other resemblances between the flowering process in these 2 types of plants. Among these similarities can be mentioned the induction of flowering by applied GA which produces bolting and flowering in the Shuokan chrysanthemum (10) as well as in *Rudbeckia speciosa* (19), whereas it does not cause flowering in short-day chrysanthemums (2, 12).

## SUMMARY

1. Changes in endogenous growth substances occurring during flower induction and development were investigated in 3 types of plants: a long-day plant, *Rudbeckia speciosa*, a cold-requiring plant, the Japanese chrysanthemum variety Shuokan, and a short-day plant, the Shasta chrysanthemum, using improved methods of extraction, chromatography and bioassay.

2. In stem tips of *Rudbeckia speciosa*, 3 major ($B_1$, $C_1$, $E_1$) and 3 minor ($A_1$, $D_1$, $F_1$) substances stimulating the elongation of oat 1st-internodes were separated chromatographically. Substance $C_1$ reached its peak after 1 week, substance $B_1$ after 2 weeks of long-day treatment, whereas substance $E_1$ which was thought to be responsible for bolting, appeared after 3 weeks of long days, just preceding bolting.

3. Three major growth substances ($B_2$, $C_2$, $E_2$) were found in the Shuokan Chrysanthemum. Substance $B_2$ reached its maximum 1 week after the beginning of the cold treatment, while substance $C_2$ decreased steadily during the vernalization process. Substance $E_2$ appeared after 3 weeks of cold treatment, which corresponds to the minimum vernalization period capable of inducing rapid bolting and flowering.

4. In methanolic extracts of the Shasta chrysanthemum, a strong inhibitor, present at all stages of flower development, prevented the detection of growth substances in the area between $R_f$ 0.6 and 0.9 in 80 % isopropanol, where substances of the E type are expected to move. Several other growth-promoting substances ($A_3$, $B_3$, $C_3$, $D_3$, $F_3$,) were detected on the chromatograms.

5. Preliminary data on the nature and properties of the active substances B, C and E are given.

6. These findings contribute experimental evidence for the existence of both quantitative and qualitative changes in the pattern of endogenous growth substances during flower induction and flower development.

Grateful acknowledgment is made to Mrs. Colette Nitsch for her invaluable help during the course of these experiments.

This work was supported in part by an assistantship given by the Department of Floriculture and Ornamental Horticulture of Cornell University, by the National Science Foundation (Grant G-4046) and by the National Institutes of Health, Bethesda, Maryland (Grant RG-4840).

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# INVERSE EFFECTS OF GIBBERELLIN ON PEROXIDASE ACTIVITY AND GROWTH IN DWARF STRAINS OF PEAS AND CORN [1]

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Several investigations have indicated that plants possessing a dwarf habit of growth show a greater peroxidase activity than their normal counterparts (4, 8). This seems to be true even where the plants are otherwise genetically identical. Thus, in a cross of Phaseolus yielding both dwarf and giant progeny, the dwarfs had a greater and the giants a lesser peroxidase activity than the normal parent types (4). In a series of hybrids in Epilobium the increase in peroxidase activity paralleled the reduction in height (6). In view of the fact that gibberellic acid (GA) is known to change the phenotype of certain strains of dwarf peas and corn to the normal (1, 5), it was of interest to see whether such dwarfs treated with GA would respond by manifesting a lower peroxidase activity as well as an increased height. Such inverse effects of GA on peroxidase activity and growth were obtained in these experiments.

## METHODS AND MATERIALS

The pea varieties used were obtained from Associated Seed Growers, Inc., New Haven, Connecticut. The dwarf pea, Progress # 9, responds well to GA, but its genetic differences from the tall type, Alaska, are partially unknown and probably complex. The corn used was a segregating population (kindly provided by Dr. Bernard O. Phinney of the University of California at Los Angeles) which yielded 25% dwarf-1 mutants, which differ from the tall plants by a single gene (5). The seeds were sown in vermiculite in 4 × 4-inch plastic containers and automatically subirrigated at 12-hour intervals with a solution of Hyponex (a complete commercial salt mixture for plant nutrition sold by the Hydroponics Chemical Company, Copley, Ohio) 120 g/100 l. The plants received a photoperiod of 16 hours and a light intensity of 1200 ft-c provided by mixed fluorescent and incandescent lights. The peas were grown at 17° C and the corn at 23° C.

Fourteen days after planting the peas were treated with gibberellic acid. One microgram GA (kindly supplied by Dr. P. W. Brian, of Imperial Chemical Industries) in 0.003 ml ethanol was applied to the stipules enclosing the 5th internode. At various times after treatment the plants were harvested and the 5th internodes excised and ground with a chilled mortar and pestle in 0.025 M, pH 6.1 $KH_2PO_4$–$Na_2HPO_4$ buffer (1 g fresh weight converted to 10 ml of brei). The homogenates were then stored in the frozen condition in Lusteroid centrifuge tubes until further use. The peroxidase activity was unchanged by such storage.

Seven days after planting the corn was treated with 1 μg of GA. This was applied in 0.003 ml of ethanol to the tip of the 1st leaf as it emerged from the coleoptile. Two and four days after treatment the plants were harvested and sections of the basal third of the 1st leaf sheath excised and homogenized as above. In each experiment segments from 20 to 30 plants were combined into 1 homogenate.

The tissue homogenate was centrifuged at 2000 G for 10 minutes and the supernatant made up to standard volume and used for peroxidase and protein nitrogen determinations. Duplicate determinations of the specific activity were made on each homogenate. Peroxidase determinations were made by the technique of Siegel and Galston (7). An aliquot of the homogenate was placed in a colorimeter tube; 0.1 ml of 0.5 M pyrogallol and sufficient buffer were added to bring the volume to 9.0 ml; at time zero 1.0 ml of $5 \times 10^{-3}$ M $H_2O_2$ was added and the tube rapidly inverted. The formation of purpurogallin was followed in the colorimeter with readings at 20, 40 and 60 seconds and the activity taken as the rate of color formation over 1 minute since the rate was linear within this time. The protein nitrogen determination was carried out after the method of Galston and Dalberg (2). The protein was precipitated with trichloroacetic acid and subjected to a micro-Kjeldahl digestion followed by Nesslerization.

## RESULTS

The effects of GA on growth and peroxidase activities of the dwarf pea internode and dwarf corn sheath are shown in table I. All activities are expressed as micromoles of purpurogallin formed per minute per microgram protein nitrogen. In the dwarf pea, Progress # 9, plants treated with GA showed a peroxidase activity depressed to 70% that of the untreated controls. This difference is significant at the 1% level 2 days after GA application. This difference between controls and GA-treated plants was evident 24 hours after treatment and persisted through the 4 days of the experiment, although the peroxidase level of the internode changed as it elongated and matured. In the tall pea, Alaska,

[1] Received October 24, 1958.
[2] Predoctoral Fellow of the National Science Foundation.

plants treated with GA showed neither an increased internode length nor a lower peroxidase level. These peroxidase activities were not changed by dialysis, indicating that the differences in activity were not due to dialyzable inhibitors or cofactors.

The peroxidase activity of dwarf-1 corn plants is higher than that of normal plants, the difference being greatest in the mature tissues of the leaf sheath. Treatment with GA lowers the peroxidase activity of the dwarf to that of the normal. A comparison of treated and control dwarf leaf sheaths 2 and 4 days after GA application shows that the peroxidase level rises in both, but that the GA-induced difference becomes greater with time. After 4 days, the differences are significant at the 1 % level (table I). As with the peas, dialysis of the homogenate did not change the peroxidase activity.

Although treatment with GA increased the length of the normal leaf sheath, it produced no significant effect on peroxidase level. GA was also without effect on the peroxidase level of the unexpanded sheath of the 2nd leaf of dwarf plants 4 days after treatment, implying that GA effects on peroxidase activity are exerted, during active growth of dwarf cells.

## Discussion

The pea internode and corn leaf sheath both show a reduced length in the dwarf varieties used here. Upon treatment with GA both these tissues show a marked elongation and decreased peroxidase. Since peroxidase activities were based upon the amount of protein nitrogen present and dialysis did not change the activities, it is assumed that the differences found in activity reflect differences in enzyme concentration and not the presence of dialyzable cofactors or inhibitors.

In the dwarf-1 mutant of corn a single genetic factor results in both decreased size and increased peroxidase content. This is in agreement with previous investigations which found that dwarf mutants of Phaseolus, Zea, Nicotiana, and Tropaeolum contained greater amounts of peroxidase than the taller plants (4, 8). In the present investigation of the peroxidase levels of 7 pea varieties, only in the variety Progress # 9, was there a correlation between increased peroxidase level and decreased size. Genetic control of internode length in peas is complex, and since the precise genetic characterization of the varieties used was not available, it is not possible to relate either dwarfism or peroxidase activity in these peas to a single gene.

The application of gibberellic acid to the dwarf corn made the dwarf phenotypically normal not only with respect to height but also with respect to peroxidase level. During the elongation and maturation of the leaf sheath the peroxidase level rises. The lower peroxidase level of the GA treated plants appears, in this instance, to be due to an inhibition of this increase.

The question may legitimately be raised whether the GA induced depression (or prevention of increase) of peroxidase activity is causally connected with the increased growth, or whether it is merely one of many biochemical consequences of the altered growth rate. This question cannot be answered at the moment. All that can be said is that the observed depression of peroxidase activity relative to the untreated controls occurs concurrently with the observed increases in growth.

In the normal corn, GA produced some increase in length but did not affect the peroxidase level. This indicates that growth rate may be controlled by a multiplicity of biochemical systems, only one of which is related to the peroxidase changes noted, and affected in the dwarf by treatment with GA.

Table I

Effect of GA Application on the Growth and Peroxidase Activities of Peas and Corn *

| Plant | GA treatment | Length of corn leaf sheath or pea internode in mm | | Peroxidase activity in mμM purpurogallin/μg prot N/min | |
|---|---|---|---|---|---|
| | | 2 days | 4 days | 2 days | 4 days |
| Dwarf corn | − | 14** | 20 | 52† | 73 |
| " | + | 32 | 44 | 46 | 56 |
| Tall corn | − | 42 | 53 | 47 | 57 |
| " | + | 54 | 64 | 44 | 54 |
| Dwarf pea (Progress # 9) | − | 5.3‡ | | 38†† | |
| " | + | 9.3 | | 27 | |
| Tall pea (Alaska) | − | 21.3 | | 21 | |
| " | + | 21.8 | | 19 | |

* Each value is the mean of at least 3 experiments.

** Confidence limits for corn leaf sheath lengths ± 8.0 % (P=0.05), ± 11 % (P=0.01)

† Confidence limits for corn peroxidase activity ± 8.5 % (P=0.05), ± 12 % (P=0.01)

‡ Confidence limits for pea internode lengths ± 9.7 % (P=0.05), ± 12 % (P=0.01)

†† Confidence limits for pea peroxidase activity ± 12 % (P=0.05), ± 21 % (P=0.01)

One other group of investigators has reported that treatment with gibberellin increases the peroxidase level (3). These investigators used guaiacol as a substrate. In repeating our work with this substrate we were able to confirm their results. Thus, it appears that the effect of GA on peroxidases involves a change in substrate specificity such that there is lower activity toward pyrogallol and higher activity toward guaiacol.

## Summary

In studies on the growing zone of the sheath of the 1st leaf of corn, it was found that the dwarf-1 mutant has a greater level of peroxidase activity than the normal wild type. Treatment with GA increases the growth rate and decreases the peroxidase activity per unit protein nitrogen of the dwarf. Although GA increases the growth rate of the normal, it has no effect upon the peroxidase level.

Using the 5th internode of the pea it was found that the dwarf variety Progress # 9 has a greater level of peroxidase activity than the tall variety, Alaska. Treatment with GA increases the growth rate and decreases the peroxidase level of this dwarf. In Alaska, a tall variety of pea, GA affected neither the growth rate nor the peroxidase level.

The authors wish to thank Dr. Chester I. Bliss for his advice on the statistical treatment and presentation of the data.

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# EFFECT OF LIGHT UPON THE BEHAVIOR OF CITRIC ACID IN LEAVES OF BRYOPHYLLUM CALYCINUM SALISB.[1]

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Although there is an extensive literature on the diurnal variation of acidity in leaves of plants of the family Crassulaceae, most discussions of the problem have been limited to a consideration of the phenomenal rhythmic changes in the amounts of malic acid present. Only occasionally has attention been paid to the closely similar behavior of citric acid, although Guthrie (2) pointed out in 1936 that citric acid may increase 5-fold in leaves of *Bryophyllum calycinum* Salisb. during the night, and that this change accounts for about one fourth of the diurnal change in titratable acidity. Guthrie made use of the gravimetric pentabromoacetone method of Hartmann and Hillig (4, 5) to determine citric acid, but it was not until rapid small-scale volumetric or colorimetric modifications of this fundamental method (3, 8) were developed that the metabolism of citric acid in plant tissues could be conveniently studied.

Guthrie's observations on the behavior of citric acid in Bryophyllum leaves have been confirmed in general outline by subsequent workers (1, 9), and no instance has been found in the experience of this laboratory of a failure of citric acid to increase when the leaves of this plant are maintained in darkness. However, as was pointed out in a recent paper (10), the converse experiment in which leaves picked at sunrise, when they contain a high level of citric acid, are exposed to light has revealed at least 2 instances in which citric acid failed to diminish in the anticipated manner. Notwithstanding this, malic acid in the same samples behaved in the normal way. A distinction could apparently be drawn between the effect of artificial illumination under fluorescent lights and the

[1] Received, October 27, 1958.

effect of exposure to daylight in a greenhouse. Under fluorescent light, the citric acid content of the leaves underwent little change; under greenhouse conditions, it diminished fairly rapidly. The temperature of the leaves was quite different in the two situations, for, during a hot summer day, the temperature of the greenhouse might rise to 38° C or higher, while the leaves under the fluorescent lights were maintained at close to 22° C. Furthermore, there were obvious differences in the quality and intensity of the radiation to which the leaves were exposed. It has seemed essential therefore to examine the citric acid content of Bryophyllum leaves exposed to light under a variety of conditions, for the evidence at hand makes it abundantly clear that the behavior of this acid does not always conform in detail with that of malic acid under similar circumstances.

## Experimental Procedure

Two sets of 10 samples each were collected by the statistical method (11) respectively on June 28, 1956 and May 29, 1957 from well grown *B. calycinum* plants raised in the greenhouse on soil. All plants were derived from the clone studied in this laboratory for many years. The top 3 pairs of well-expanded but young 5-leaflet leaves were used, and each sample contained 60 leaflets derived from 20 plants so chosen that each plant and each leaflet position on the plant were equally represented in each sample. Collection was begun at about 20 minutes before sunrise. The zero hour was taken as 6:00 A.M. when all leaves were in position in the culture troughs and the initial control sample was placed in the drying oven. The general technique of such culture experiments has been described in previous papers (9, 10) in which the methods of preparing the samples for analysis and references to the analytical methods used are also given.

Experiments A and E (table II) were carried out in the greenhouse, and Experiments B, C, G, and H in the light rooms of the Department of Botany in the Gibbs Laboratory of Yale University. We are deeply indebted to Professor A. W. Galston for the use of this excellent equipment. Experiment D was carried out in the temperature-controlled light room of the Department of Genetics of this Station.

Evidence for the uniformity of the initial composition within each of the sets of samples is given in table I. The analytical data are calculated in terms of an initial fresh weight of 1 kg of leaves per sample, and it is clear that the composition of each set of samples was acceptably uniform, although the 2 sets differed from each other. The leaves of the 1957 samples were somewhat younger and less fully developed than those collected in 1956. The data for isocitric acid are included to demonstrate that this component did not change significantly in response to the treatment of the samples if the sampling and analytical errors, both of which are of the order of 2%, are taken into consideration. The data for organic solids show that the net increase from the effects of photosynthesis was too small to be detected with certainty.

Table II gives the details of the conditions under which the leaves were illuminated and the results of the analytical determinations of organic acids and starch. The 1st and 11th lines show the initial composition of the leaves of the 2 sets of samples. The 2nd and 12th lines show the effect upon the composition of the leaves of exposure to darkness for 11 (or 10) hours in addition to the approximately 9 hours of darkness during the night before collection. These observations serve as controls, and demonstrate that the behavior in darkness of the organic acids and starch of these samples was normal.

The greenhouse in which the leaves of Experiments A and E were treated had been given a coat of whitewash with a little green dye to cut down the intensity and diffuse the light. Although the light was dim at the start of Experiment A, the intensity had increased to 1000 ft-c by 7:30 A.M. and did not drop to this level again until the last few minutes of the 11-hour period when a thunder shower occurred; during most of the day, the intensity was well above 3000 ft-c. The lighting equipment at the Gibbs Laboratory is provided with fluorescent bulbs described as "cool white" and "warm white" which deliver light over a broad range of wave lengths. Such plants as peas have been raised to maturity and have set seed under these lights. The room used for Experiment D provided light at a slightly lower intensity. However, these lights have been demonstrated to be com-

Table I

Analytical Data on 2 Sets of 10 Samples Each of Leaflets of Bryophyllum calycinum Collected by the Statistical Method

| Analytical determination | Samples 1956 A | | | Samples 1957 A | | |
|---|---|---|---|---|---|---|
| | Mean | S.D.* | C.V.* | Mean | S.D.* | C.V.* |
| Initial fresh weight, g/sample | 301.8 | ±3.85 | 2.3 | 174.0 | ±3.23 | 1.9 |
| Total nitrogen, g/kg | 2.03 | ±0.03 | 1.5 | 2.40 | ±0.04 | 1.8 |
| Protein nitrogen, g/kg | 1.63 | ±0.02 | 1.2 | 1.98 | ±0.05 | 2.3 |
| Ash, g/kg | 10.56 | ±0.12 | 1.2 | 12.97 | ±0.26 | 2.0 |
| Isocitric acid, meq/kg | 184.5 | ±5.3 | 2.9 | 255.3 | ±7.4 | 2.9 |
| Equilibrated dry weight, g/kg | 92.3 | ±1.0 | 1.0 | 115.7 | ±2.3 | 2.0 |
| Organic solids, g/kg | 75.45 | ±0.77 | 1.1 | 91.68 | ±1.94 | 2.1 |

* S.D., standard deviation; C.V., coefficient of variation as percentage.
All analytical data expressed in terms of 1 kg of initial fresh weight of leaves.

pletely adequate for the formation of starch in Bryophyllum leaves at a high rate (see data for Experiment 1952-A in reference (10)).

The results of Experiment A in the greenhouse, in comparison with those of Experiments B and C at 21° and 41° C, raised the question whether the quality of the light from the fluorescent bulbs at the Gibbs Laboratory was adequate to bring about a rapid utilization of citric acid and, accordingly, in 1957, a 2nd set of experiments was carried out in 1 of which (Experiment H) the fluorescent light was supplemented with four 200-watt frosted incandescent bulbs.

The leaves which were cultured out of doors in Experiment F were partially shaded until 8:30 A.M. when the light intensity was 1200 ft-c, but were subsequently in full sunlight with only light clouds toward the end of the treatment when the intensity had dropped to about 5700 ft-c.

The temperature in the greenhouse was only 10° C at 6:00 A.M. of the morning of the 1957 experiment, but had risen to 20° C by 9:00 A.M. The highest temperature attained was 39° C at 2:00 P.M. The out of door temperatures on the same day (Experiment F) were about the same except that the maximum was only 32° C at 2:00 P.M. The temperatures of the light rooms were controlled within a degree or two of the figures shown in table II with the exception that the temperature during Experiment H presumably ranged between 23° C, the controlled temperature of the air of the room, and 31°, the temperature attained fairly promptly by the water in the culture troughs under the fluorescent and incandescent lights.

## Experimental Results

The data for the organic acids and starch in table II show the effects produced under the several experimental conditions. Both of the control samples behaved normally in darkness. Malic acid was present at a high level at sunrise, and had diminished slightly after an additional 10 or 11 hours of darkness. It presumably increased a little during the 1st few hours, but it has long been known that, after Bryophyllum leaves have been in darkness for about 12 hours, a slow loss of malic acid begins. A curve which shows this behavior in some detail is given in figure 2 of reference (10). The present observations at 11 (or 10) hours were made at times such that the loss of malic acid had presumably only recently started.

Citric acid increased by 41 % of the quantity present at sunrise during the 11 hour additional period of darkness in the control sample of the 1956 experiment, and by 93 % during 10 hours in that of the 1957 experiment. The enzyme systems concerned with the

Table II

Effects Upon Organic Acids and Starch of Illumination of Leaflets of Bryophyllum calycinum Under Various Conditions

| Samples | line | Conditions of culture | Experiment | Temp | Light intensity | Time | Malic acid | Citric acid | Isocitric acid | Starch | pH |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *° C* | *ft-c* | *hr* | *meq/kg* | *meq/kg* | *meq/kg* | *g/kg* | |
| 1956 A | 1 | Initial fresh leaf | | | | 0 | 192 | 60.6 | 180 | 6.8 | 4.01 |
| " | 2 | Darkness | | 23 | 0 | 11 | −13 | +25.1 | +10 | −2.3 | 4.00 |
| " | 3 | Greenhouse | A | 20 to 37 | 180 to 4000 | 5 | −91 | −8.8 | +2 | +4.8 | 4.48 |
| " | 4 | " | A | " | " | 11 | −165 | −44.8 | +6 | +8.7 | 5.61 |
| " | 5 | Fluorescent; warm white and cool white | B | 21 to 23 | 1400 | 5 | −46 | −4.8 | −4 | +2.7 | 4.18 |
| " | 6 | " " | B | " | " | 11 | −147 | −15.7 | +16 | +6.5 | 4.87 |
| " | 7 | Fluorescent; warm white and cool white | C | 41 to 42 | 1400 | 5 | −88 | −8.1 | +4 | +3.2 | 4.48 |
| " | 8 | " " | C | " | " | 11 | −148 | −25.0 | +4 | +6.9 | 5.16 |
| " | 9 | Fluorescent; cool white | D | 20 to 23 | 1000 to 1100 | 5 | −51 | −2.4 | +5 | +2.0 | 4.22 |
| " | 10 | " | D | " | " | 11 | −153 | −20.5 | +4 | +7.2 | 5.02 |
| 1957 A | 11 | Initial fresh leaf | | | | 0 | 243 | 38.3 | 257 | 11.2 | 4.05 |
| " | 12 | Darkness | | 22 | 0 | 10 | −14 | +35.6 | −4 | −4.9 | 4.00 |
| " | 13 | Greenhouse | E | 10 to 39 | 200 to 4600 | 5 | −48 | +5.3 | −13 | +2.5 | 4.19 |
| " | 14 | " | E | " | " | 10 | −225 | −22.2 | −7 | +10.5 | 5.62 |
| " | 15 | Out of doors sunlight | F | 15 to 32 | 500 to 7800 | 5 | −53 | +4.6 | −11 | +2.7 | 4.21 |
| " | 16 | " | F | " | " | 10 | −222 | −19.0 | +6 | +10.4 | 5.52 |
| " | 17 | Fluorescent; warm white and cool white | G | 23 | 1300 | 5 | −89 | +2.8 | −2 | +3.1 | 4.28 |
| " | 18 | " " | G | " | " | 10 | −203 | −12.8 | +3 | +8.6 | 5.08 |
| " | 19 | Fluorescent and incandescent | H | 31 | 2000 | 5 | −175 | −2.7 | +1 | +8.1 | 4.88 |
| " | 20 | " " | H | " | " | 10 | −227 | −26.5 | +11 | +12.2 | 5.67 |

All analytical data expressed in terms of 1 kg of initial fresh weight of leaves. The plus and minus signs show the changes from the initial composition at each point of observation.

TABLE III

COEFFICIENTS OF CORRELATION BETWEEN CERTAIN COMPONENTS OF LEAFLETS OF BRYOPHYLLUM CALYCINUM ILLUMINATED UNDER VARIOUS CONDITIONS

| | | |
|---|---|---|
| 1956 A | Malic acid vs. starch | −0.962 |
| | Citric acid vs. starch | −0.939 |
| | Total non-volatile acids vs. pH | −0.960 |
| 1957 A | Malic acid vs. starch | −0.951 |
| | Citric acid vs. starch | −0.943 |
| | Total non-volatile acids vs. pH | −0.966 |

($r = 0.872$ for $P = 0.001$ with 8 degrees of freedom)

synthesis of citric acid in darkness in these samples were thus fully active. The starch content of the 1956 samples was quite low at sunrise, but diminished appreciably during the additional period in darkness. It was higher at sunrise in the 1957 samples than in those examined in 1956 and diminished even more notably during the additional treatment in darkness. The enzyme mechanisms concerned with the metabolism of starch in these samples were thus also normally active.

Before considering the effect of illumination under various conditions upon the organic acid and starch content of these 2 sets of samples, it is desirable to point out the close mathematical relationship between certain groups of the data. In table III are shown the coefficients of correlation between malic acid and starch, and between citric acid and starch, as calculated from the quantities of these components ultimately present in each of the 2 sets of 10 samples. The coefficient of correlation between the total non-volatile organic acids and the pH is also shown. It is obvious, from the chemical point of view, that the change in the pH of the water extracts of such samples of leaves should be closely related to the change in their content of organic acids, for the samples were initially identical in composition. The calculation shows that this is indeed true; the probability is far less than 1 in 1000 that this relationship is one of chance. The important point, however, is that the relationship between the quantities of malic acid and starch, and that between citric acid and starch are almost equally close; the coefficients of correlation are almost identical with those for the organic acids versus pH. Furthermore, this relationship holds for both sets of samples, and similarly high coefficients have been found for the data obtained from many other sets treated in various ways (9, 10). The interpretation of such mathematical results as evidence for a chemical relationship seems justified.

In order to simplify the consideration of the data in table II which show the total changes in the quantities of organic acids that occurred at 2 points of time under the influence of various conditions of illumination, the rates at which malic acid and citric acid changed in the 2 successive intervals of observation have been calculated and are given in table IV. Such calculations assume that the reactions followed straight line functions during the period of observation, which is almost certainly not true. However, the figures are valid when considered as mean rates within the given intervals of time.

RATE OF CHANGE OF MALIC ACID: Under the several conditions of illumination, from 75 to 86 % of the malic acid of the 1956 samples disappeared during 11 hours of exposure to light. However, in the 1957 samples, the enzyme systems concerned with the metabolism of malic acid appear to have been somewhat more vigorous in their activity, for from 92 to 93 % of the malic acid disappeared in 10 hours although the initial content was higher (table II). Regardless of the differences in the conditions under which the leaves were exposed to light, it is clear,

TABLE IV

RATES OF CHANGE OF MALIC ACID AND CITRIC ACIDS IN LEAFLETS OF BRYOPHYLLUM CALYCINUM ILLUMINATED UNDER VARIOUS CONDITIONS

| SAMPLES | CONDITIONS OF CULTURE | EXPERIMENT | TEMP. | LIGHT INTENSITY | RATE OF CHANGE OF MALIC ACID | | | RATE OF CHANGE OF CITRIC ACID | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | 0–5 hr | 5–11 hr | 0–11 hr | 0–5 hr | 5–11 hr | 0–11 hr |
| | | | 0°C | *ft-c* | *meq/hr* | *meq/hr* | *meq/hr* | *meq/hr* | *meq/hr* | *meq/hr* |
| 1956 A | Greenhouse | A | 20 to 37 | 180 to 4000 | −18.3 | −12.3 | −15.0 | −1.8 | −6.0 | −4.1 |
| " | Fluorescent light | B | 21 to 23 | 1400 | −9.1 | −16.8 | −13.3 | −0.9 | −1.8 | −1.4 |
| " | Fluorescent light | C | 41 to 42 | 1400 | −17.6 | −10.0 | −13.4 | −1.6 | −2.8 | −2.3 |
| " | Fluorescent light | D | 20 to 23 | 1000 to 1100 | −10.2 | −17.1 | −13.9 | −0.5 | −3.0 | −1.9 |
| " | Darkness | | 23 | 0 | | | −1.1 | | | +2.3 |
| | | | | | 0–5 hr | 5–10 hr | 0–10 hr | 0–5 hr | 5–10 hr | 0–10 hr |
| 1957 A | Greenhouse | E | 10 to 39 | 200 to 4600 | −9.7 | −35.2 | −22.4 | +1.1 | −5.5 | −2.2 |
| " | Out of doors | F | 15 to 32 | 500 to 7800 | −10.6 | −33.9 | −22.2 | +0.9 | −4.7 | −1.9 |
| " | Fluorescent light | G | 23 | 1300 | −17.7 | −22.9 | −20.3 | +0.6 | −3.1 | −1.3 |
| " | Fluorescent and incandescent | H | 31 | 2000 | −34.9 | −10.5 | −22.7 | −0.5 | −4.7 | −2.7 |
| " | Darkness | | 22 | 0 | | | −1.4 | | | +3.6 |

from the rates calculated for the entire 11 (or 10) hour period of observation (column 7, table IV), that the overall net rate at which malic acid was metabolized was essentially constant within each set of samples, although the rates differed between the 2 sets. Furthermore, the mean net rates of −14 meq per hour for the 4 groups of 1956 samples and of −22 meq per hour for the 4 groups of 1957 samples are within the range of rates that have been calculated for a number of other sets of samples (see table III of reference 10). The precise rate observed for a given set of samples will depend, among other things, upon the level of malic acid present in the leaves at sunrise. In the experience of this laboratory, this may range from a little less than 200 meq per kg to as much as 300 meq per kg, and, during a period of about 10 hours in light, by far the greater part of it disappears.

The effect of the different conditions under which the leaves were illuminated can best be perceived if the rates during the 1st 5 hours of illumination (column 5, table IV) are compared with the rates during the subsequent 6 (or 5) hours (column 6). The 1956 samples exposed in the greenhouse (Experiment A) on a warm day lost malic acid rapidly in the 1st interval, and the rate of loss compares closely with that from the leaves under fluorescent light at 41° C (Experiment C). Since more than one half of the total change occurred in this interval, the mean rate during the 2nd period of 6 hours was smaller. The conditions under which Experiments B and D were carried out were similar with respect to temperature, and the light intensity was not widely different. As a result, the respective mean rates of loss of malic acid in the successive intervals were essentially the same.

The conditions in the greenhouse during Experiment E were quite different from those of Experiment A. During the 1st few hours, the temperature was low, and the rate of loss of malic acid was correspondingly slow. The results from Experiment F, in which the leaves were out of doors, were similar. In both of these tests, the temperature rose rapidly in midmorning, and the rates at which malic acid was utilized during the 2nd interval of observation were notably increased.

The conditions to which the samples of Experiments B and G were exposed were nearly identical; the same illuminating equipment at almost the same temperature was employed. Although the rates of loss of malic acid were different for the 2 sets of samples, in both experiments the rate during the 2nd interval was greater than that in the 1st; that is to say, the rate at which malic acid entered into reaction under constant conditions apparently increased after from one quarter to one third of it had disappeared. The addition of incandescent lights to the fluorescent lights (Experiment H) raised the temperature to about that reached out of doors at noontime, as well as providing a greater intensity and, as a result, the rate of loss of malic acid during the 1st 5 hours was about the same as the rates observed in the 2nd intervals of Experiments E and F. Since about 72 % of the malic acid had disappeared at the end of 5 hours, the subsequent rate of loss in Experiment H was low.

The data for the behavior of malic acid under the conditions of the present tests suggest that the rates of loss under either natural or artificial illumination are strongly affected by the temperature. Although there were undoubtedly differences in the distribution of energy with respect to the wave length of the light, no clearcut instance can be pointed out in which the quality of the light dominated the situation.

Rate of Change of Citric Acid: The 1956 samples initially contained 60.6 meq of citric acid per kg, a concentration commonly found in Bryophyllum leaves picked at sunrise. The 1957 samples contained the somewhat lower concentration of 38.3 meq per kg. The data in table II show that, in the 1956 samples, citric acid diminished much less during the 1st 5 hours, regardless of the source of light, than during the subsequent period of observation. In the 1957 samples, citric acid *increased* during the 1st 5 hours in all instances save in Experiment H in which, however, it decreased by only a small amount. The behavior of citric acid thus obviously differs in certain fundamental respects from that of malic acid. When calculated for the entire period of exposure to light, the rates at which citric acid diminished in the 1956 samples (table IV, column 11) ranged from −1.4 to −4.1 meq per hour, a 3-fold variation in rate under different conditions, and a result which contrasts sharply with the relatively constant mean rate of loss of malic acid (column 7). The rate in the 1957 set of samples varied 2-fold. The behavior of citric acid thus seems to be more sensitive to light intensity and temperature than that of malic acid.

The rate of loss of citric acid in the 1st 5-hour period was about the same in Experiments A and C, both being small. The temperature of the greenhouse (Experiment A) had reached 26° C within an hour (light intensity 900 ft-c), and ranged upward to 34° at the end of 5 hours when the light intensity was 4000 ft-c. The leaves of Experiment C were held at a constant temperature of about 41° C and a light intensity of 1400 ft-c. During the subsequent 6-hour period, however, the rate of loss of citric acid from the leaves in the greenhouse was about twice that observed in the leaves under fluorescent light in the room at 41° (column 10), although the temperature in the greenhouse at no time exceeded 38° C.

The samples of Experiments B and D were exposed to closely similar and constant conditions with respect to temperature and light intensity. The rate of loss of citric acid in the 1st 5-hour period was small in both, but was a little greater in the samples at the Gibbs Laboratory (Experiment B). In the 2nd period, however, the rate of loss from the leaves in Experiment D considerably exceeded that in Experiment B. This result is the reverse of what might be expected if the quality of the light differed in the 2 locations and the advantage lay with the light equipment at the Gibbs Laboratory. The data are confus-

ing, especially in view of the similarity of the behavior of malic acid in these 2 experiments. However, if the information obtained from the 1957 samples is considered, and it is assumed that citric acid in the 1956 samples at first increased to a maximum and then decreased, the discrepancies between the calculated rates of loss under the apparently similar conditions in the 2 locations may be cleared up to some extent. Observations at shorter intervals of time will be required to establish this, but it seems likely that small differences in the intensity of the effective factor, whatever this may be, may alter the precise location of the maximal concentration of citric acid with respect to time. Accordingly, a single observation at an arbitrarily selected point may reveal an increase or a decrease. An analogous case is the observation of a decrease in malic acid in the control experiments after 11 (or 10) hours in darkness in addition to the 9-hour night period (table II). Many previous experiments (10) have shown that the content of malic acid passes through a maximum and then decreases after from 12 to 16 hours exposure of the leaves to darkness.

The behavior of citric acid in the leaves of the 1957 experiments was somewhat more uniform than in those examined in 1956. Experiments E and F in sunlight differed only in the range of temperature and light intensity to which the samples were exposed. In both, citric acid increased during the 1st 5 hours at approximately the same net rate, and in both it decreased during the 2nd 5 hours at a high rate. That the increase during the 1st 5 hours was not necessarily an effect of the broader range of wave lengths and higher intensity of the light, nor even of the higher temperature to which the leaves were exposed for a part of the time is clear from the result of Experiment G. Citric acid also increased in the leaves exposed to fluorescent light in the 1st 5 hours although at a somewhat smaller rate. However, under the continuous high temperature conditions in Experiment H and with the additional radiation from the incandescent lights, citric acid had diminished at the end of 5 hours. In all 4 experiments, citric acid diminished rapidly during the 2nd 5 hour period, although at a somewhat lower rate under the fluorescent lights.

Previous Observations on the Effect of Light on the Metabolism of Citric Acid: The data that have been presented indicate that a few hours after Bryophyllum leaves picked at sunrise have been exposed to light, a steady although slow loss of citric acid can be demonstrated to have been initiated. The rate of loss is greater in sunlight than under fluorescent light, and there is evidence that the reaction is promoted by an increase in the temperature at which the experiment is conducted. That the effect begins as soon as the leaves are exposed to light seems doubtful, for increases in the citric acid content were observed after 5 hours of illumination in some of the tests, although loss subsequently occurred. The situation is manifestly far from simple, and generalization is made even more difficult if the results of 2 earlier experiments on the behavior of the organic acids in the leaves of this species exposed to fluorescent light are considered.

In the 1st of these, which was carried out with leaves from the same clone grown in the season of 1951 (12), the intensity of the light was approximately 750 ft-c and the temperature fluctuated with the operation of the controlling equipment between 21° and 26° C. Samples taken at intervals of 5 hours from the start contained, respectively, 59, 61, 54, 53 and 53 meq of citric acid per kg of initial fresh weight. During this same period of 20 hours, malic acid diminished from 264 meq to 45 meq per kg, and starch increased from 12.7 to 29.7 g per kg. In the 2nd experiment (10) in which the intensity of the light was approximately 1100 ft-c and the temperature 20° to 22° C, the leaves contained initially 62 meq per kg of citric acid and, after 14 and 27 hours respectively, contained 64 and 65 meq per kg. The data for malic acid and starch were closely similar to those of the 1st experiment. The leaves examined in the 2nd experiment were demonstrated to retain the capacity to accumulate citric acid effectively when transferred to a dark room at the same temperature. In both experiments, the period of exposure to light was prolonged for more than 60 hours, and in both only a slow loss of the order of 10 meq of citric acid subsequently took place.

## Discussion

The data on the behavior of citric acid in Bryophyllum leaves cultured in light are admittedly confusing and, in some respects, appear to be conflicting. It is clear that the statement that the diurnal behavior of citric acid is similar to that of malic acid although at a lower level with respect to quantity is only partly true. Both acids do indeed increase at the expense of starch when the leaves are cultured in water in darkness, and the increases when expressed in relative terms are similar in magnitude; that is, under conditions such that malic acid increases 7-fold, citric acid may increase 6-fold (10). In light, however, the situation is obviously much more complex, and the outcome of a test under any arbitrarily established set of conditions is to a considerable extent unpredictable. Experiment shows that citric acid may increase or decrease, or may even remain essentially constant in concentration.

Nothing is definitely known of the precise enzymatic mechanisms whereby citric acid is either synthesized or decomposed in the cells of this species, although one would be inclined to speculate that the reactions are analogous to those of the well-known tricarboxylic acid cycle. Pending the experimental demonstration of the presence of the appropriate enzyme systems in this tissue, and of the factors which control the direction and rate of the reactions which take place, all that can at present be offered to account for what occurs is an hypothesis.

The facts available are essentially as follows: When the experimental conditions are such that a wide variation in the content of starch occurs and, at the same time, a wide variation in the opposite direction of either malic acid or citric acid or both takes place, the data for starch vs. malic acid and for starch vs. citric acid are mathematically closely correlated (9, 10, 12). It is accordingly assumed that a reversible chemical interconversion as between carbohydrate and the organic acids occurs. The 2nd fact is that conversion in the direction of starch formation requires a supply of light energy, whereas conversion in the opposite direction occurs in darkness and is presumably exergonic. A 3rd fact which may have a bearing on the present problem is that malic acid is readily converted into citric acid by enzyme systems present in the tobacco leaf, and that the reaction is, to some extent at least, reversible (13, 14). This reaction occurs when the excised leaves are cultured in water in darkness, and is promoted if the culture solution contains malate or other related organic acids. To what extent it occurs in illuminated tobacco leaves is not known since the levels of malic and citric acids present change but little under such circumstances (15).

The hypothesis that can be suggested to account to some extent for the present results on the behavior of citric acid in the Bryophyllum leaf may be set up as a series of propositions:

a) In darkness, starch undergoes a rapid series of chemical transformations the chief end-product of which is L-malic acid. This substance accumulates because the rate at which it is converted into further products is slower than the rate at which it is formed.

b) In darkness, and probably also to some extent and for a short time, in light, malic acid undergoes transformations that result in the formation of citric acid. This reaction is slow compared to Reaction a).

c) In light, Reaction a) is extensively and rapidly reversed.

d) In light, Reaction b) is also reversed, although slowly.

e) These reactions proceed continuously, the net effect being controlled by the presence or absence of light, and the net rate, as measured by the rate of accumulation of the ultimate product, is determined in the first place by the temperature and in light by the intensity, although other factors such as age and degree of development of the tissue and especially the relative concentration of the major reactants are also involved.

Each of these transformations represents the ultimate but easily observable effect of what is doubtless a long, integrated series of enzymatically catalyzed reactions in which many intermediate substances are involved. Each successive enzymatic reaction presumably proceeds at its own characteristic rate, this rate being controlled or influenced by the concentrations of the specific enzymes, and of the co-factors, substrates and, if present, inhibitors, and also by the physical conditions surrounding the tissue. Manifestly, a system so complex as this will be responsive to influences of many kinds, and some of these may well be so subtle as to escape observation and be beyond control. Accordingly, experiments may not always give the anticipated result.

If this hypothesis has merit, it should be possible to test one aspect of it by comparison of the quantities of organic acids and starch which become involved in the reactions that occur under experimental conditions. In order to avoid all considerations of specific mechanisms, these quantities are expressed as the number of millimoles of carbon in the amounts of initial and final substances which underwent chemical change. The results thus merely deal with the question whether or not the proposed hypothesis represents a possible description of what occurs.

It was shown in a recent paper (10) that, after 18 hours of culture in darkness of Bryophyllum leaves picked in the evening, the ratio of the increase of carbon combined in malic and citric acids to the loss of carbon combined in starch was 1.04. In the companion experiment in which leaves picked at sunrise were exposed to artificial light for 27 hours, the similarly calculated ratio was 1.09. Thus, during somewhat prolonged experimental periods, the behavior of the organic acids and starch conforms on the average with the requirements of the hypothesis.

The data for the composition of the leaves of the several samples in table II provide an opportunity to study the effect of relatively short periods of exposure of similar leaves to light. The data have been analyzed in 2 ways. There are 16 observations of the increase of starch and the concomitant decreases of malic and citric acid in leaves under various conditions of illumination. The total change of each of these components during the 1st 5 hours in all tests, and during 11 hours in the 1st group of 8 experiments and 10 hours in the 2nd group of 8 have been calculated in terms of the number of millimoles of carbon that were either gained or lost. The ratio of the quantity of carbon of malic acid involved to that of the starch has been computed, and also the ratio of the sum of the carbon of malic and citric acids to that of the starch. Secondly, similar calculations have been made for the changes that occurred in the 1st 5 hours and also for the changes during the succeeding 6 (or 5) hours. The data for each of these sets of calculations have been pooled, and the means and standard deviations together with the ranges are shown in table V. No correction for the amount of starch that arose from photosynthesis was applied since the change in the organic solids was too small to be detected with certainty (table I).

A further refinement has been made in the calculations shown in the last line of the table to take account of the requirements of the hypothesis that citric acid arises from malic acid. Where citric acid diminished, it is assumed that a mole for mole conversion occurred; where it increased, it is assumed, in conformity with the results of many experiments with tobacco leaves (13, 14), that 2 moles of malic acid are required for

the synthesis of 1 mole of citric acid. The effect of the refinement is to bring the mean results, whether calculated for the entire period of each of the treatments of the individual samples, or for the 2 successive periods, into agreement, the value of the ratio being approximately 1.2 ± 0.2. It seems clear, therefore, that sufficient carbon was provided by the quantities of organic acids which underwent chemical change to account for all of the starch that was formed. The hypothesis that has been set up thus describes a possible mechanism. That the ratio is somewhat greater than unity is to be anticipated since no account is taken of the respiratory loss nor of the small concentrations of intermediate substances present in the tissues. These intermediates presumably arose from transformation of malic acid.

ORGANIC ACID METABOLISM OF BRYOPHYLLUM LEAVES: The major organic acids present in Bryophyllum leaves display 3 quite different orders of reactivity in terms of their rate of response to alterations in the physical conditions that surround the plant. Isocitric acid may be present at a level of from 180 to 250 meq per kg of fresh weight (table II), and does not in general respond to any detectable extent to alternation of light and darkness (9). Only one instance has been observed in which isocitric acid became involved in the organic acid metabolism; it diminished significantly during the early hours of an experiment in which a set of samples of leaves picked at sunrise which chanced to be abnormally low in starch content was subjected to culture in darkness (16, 17); in all other experiments carried out in this laboratory, isocitric acid has remained essentially unchanged for many hours of treatment either in light or in darkness.

Citric acid is, however, responsive to changes in illumination, but the rate at which it enters into reaction is sluggish in comparison with the rate at which malic acid undergoes change. This is well brought out by the data of table IV, and was also emphasized by the results of a study of the effects of temperature upon the organic acid metabolism (18). The rate at which citric acid was formed in the tissues in darkness at 6° C was only a little more than one half that at 24°, although there was little effect upon the rates at which malic acid was synthesized at these 2 temperatures during the 1st 18 hours of the test.

At the 3rd level of reactivity is malic acid, the classical example of an organic acid which fluctuates rapidly between wide limits in response to alternations of light and darkness, starch being produced when it decreases in light and decreasing again when malic acid is formed in darkness.

If it is assumed that citric acid arises in the Bryophyllum leaf by transformations of malic acid analogous to those which take place in tobacco leaves during culture in darkness, it becomes possible to account to some extent for the observations on the organic acid metabolism of this plant. Although many factors doubtless enter into the control of the direction and rate of the various reactions that take place, it may be assumed that the dominant factor under any given set of physical conditions is the relative proportions of the several reactants present. Thus at daybreak, when the concentration of malic acid is high, the major sequence of reactions which occurs leads to the formation of starch. However, if the hypothesis that has been suggested is valid, the level of citric acid present at daybreak is also determined by the high concentration of malic acid, for a continuous although slow conversion of malic acid into citric acid presumably takes place during the night. There is no reason to suppose that light would inhibit this reaction and so, for a short time after the leaves are illuminated, part of the malic acid may continue to be converted into citric acid. An increase of citric acid was observed in the early stage of 3 of the experiments in table II. Subsequently, as the concentration of malic acid drops, the reactions which maintain citric acid at a high level

TABLE V

RELATIONSHIP BETWEEN THE CARBON OF THE STARCH WHICH DISAPPEARED AND THE CARBON OF THE MALIC AND CITRIC ACIDS WHICH WERE FORMED DURING THE CULTURE OF LEAVES OF B. CALYCINUM UNDER VARIOUS CONDITIONS OF ILLUMINATION

| | Pooled data for effects after 5 hours and after 11 (10) hours | | | | Pooled data for effects after 5 hours and during the succeeding 6 (5) hours | | | |
|---|---|---|---|---|---|---|---|---|
| | *Mean* | *S.D.* | *C.V. %* | *Range* | *Mean* | *S.D.* | *C.V. %* | *Range* |
| Ratio of carbon of malic acid lost to carbon of starch formed | 1.17 | ±0.18 | 15 | 0.92 to 1.55 | 1.27 | ±0.22 | 20 | 0.69 to 1.55 |
| Ration of sum of carbon of malic and citric acids lost to carbon of starch formed | 1.26 | ±0.21 | 17 | 0.94 to 1.77 | 1.24 | ±0.22 | 17 | 0.93 to 1.60 |
| Ratio of sum of carbon of malic and citric acids lost to carbon of starch formed: refined data* | 1.22 | ±0.19 | 16 | 0.89 to 1.53 | 1.21 | ±0.21 | 18 | 0.91 to 1.57 |

* If the citric acid which disappears is converted to malic acid, mole for mole, and this in turn to starch, only 2/3 of the carbon of the citric acid is contributed to the starch. Thus, to calculate the amount of carbon which may have undergone this change, the change in citric acid expressed in meq. is multiplied by 1.33 to give millimoles of carbon. In the 3 instances where citric acid increased (Experiments E, F, and G after 5 hours), it is assumed that 2 moles of malic acid were used for the synthesis of 1 mole of citric acid (14).

are no longer effective, and citric acid accordingly diminishes in concentration. If this is a correct view of the situation, it becomes clear why citric acid in general follows the behavior of malic acid and responds, although sluggishly, to the alternations of light and darkness; for in darkness, as starch diminishes and malic acid increases, citric acid would slowly accumulate in step with this increase.

Whether or not this view of the situation can be extended to account for the apparent stability of isocitric acid in Bryophyllum leaves is problematical. When considered in terms of their chemical relationships, isocitric acid would be assumed to arise from citric acid by reactions analogous to those promoted by the well-known enzyme aconitase of mammalian tissues. At present, however, there is no evidence to support the view that an analogous mechanism is responsible for the synthesis of isocitric acid in Crassulacean plants. In fact, the stability of isocitric acid in Bryophyllum leaves under conditions such that both malic acid and citric acid undergo wide fluctuations in concentration might be cited in favor of some mechanism other than an aconitase reaction of normal type to account for the accumulation of the extraordinary concentrations of isocitric acid found in many species of this family (19). In *B. calycinum*, this substance may be present at sunrise at a concentration 3 to 6 times that of citric acid (table II); after exposure of the leaves to light, the ratio may increase to from 11 to 16 (Experiments A and E, table II). The existence of such relative concentrations of these 2 acids in a system which contains an aconitase similar to the enzyme of mammalian tissues is clearly unlikely, for in experiments in vitro, the relative proportions of citric to *cis*-aconitic to isocitric acid at equilibrium in the presence of aconitase are approximately as 89 : 4 : 7 (6). If isocitric acid does indeed arise from the activity of an aconitase, some additional mechanism must be present to isolate it from the sphere of activity of the enzyme as rapidly as it is formed. An explanation either in chemical or physiological terms of the accumulation of isocitric acid in Crassulacean plants is obviously still to be obtained.

## Summary

An examination of the effect upon the citric acid content of leaves of *Bryophyllum calycinum* Salisb. picked at daybreak of exposure to sunlight and to artificial light under various conditions has shown that citric acid may at first increase in concentration although subsequently it decreases. The diurnal behavior of citric acid is thus not identical with that of malic acid.

It is suggested that citric acid is formed in this species by reactions to which malic acid makes a major contribution, and that these reactions are, at least to some extent, reversible, the direction of the reactions being controlled to a large extent by the relative proportions of the 2 substances present. The concentration of citric acid is thus responsive to the diurnal rise and fall of the concentration of malic acid. This view of the situation accounts not only for the diurnal fluctuations of citric acid, but also for the temporary increase of citric acid shortly after daybreak while the relative concentration of malic acid is high.

An explanation of the accumulation of isocitric acid in high concentrations in Crassulacean plants remains to be obtained.

Grateful acknowledgement is made to Dr. Israel Zelitch for helpful discussion, to Katherine A. Clark and Laurence S. Nolan for technical assistance and to the National Science Foundation for a grant which supported a part of this work.

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# RESPIRATORY AND ASSOCIATED RESPONSES OF CARROT DISCS TO SUBSTITUTED PHENOLS [1]

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The striking physiological and biochemical effects of the classical uncoupling agent 2,4-dinitrophenol (DNP) are now well known, (4, 5, 15) though still imperfectly understood. The pertinent work with plant materials has been reviewed by Simon (21) and Laties (16). Stenlid, who carried out some of the earliest work on plants, reported that 5 other nitrophenols brought about similar respiratory responses to those induced by DNP, and, like DNP, inhibited glucose uptake by wheat roots (24). The behavior of the nitrophenols was compared to that of azide (23) and later to that of methylene blue (25).

In this paper quantitative comparisons have been made of the effects of a series of $NO_2$-, Cl-, and Br-substituted phenols on respiration and concommitant glucose uptake. In addition, the effects of these compounds on mitochondrial oxidations have been determined. The conclusion is drawn that, with different degrees of efficiency, all of the substituted phenols acted as uncoupling agents.

Observations on the effects of the phenols on $C^{14}O_2$-output from glucose-$C^{14}$ are also presented and discussed in relation to the question raised recently by Porter and Runeckles (19) of a possible shift in respiratory substrate resulting from DNP treatment.

## MATERIALS AND METHODS

Carrots of 25 to 30 mm average diameter were purchased locally and stored at 3 to 5° C for at least a week before use. Discs of about 0.3 mm thickness and 7 mm diameter were prepared, washed quickly in 3 changes of distilled water, dried lightly, and separated into weighed samples. The various phenols and other reagents were of the best grade commercially available, and were used without further purification. Radioactive glucose, uniformly labeled with $C^{14}$ was prepared by hydrolysis and chromatographic separation from a sample of sucrose-$C^{14}$ produced during photosynthesis in $C^{14}O_2$ and generously supplied by Dr. H. R. Garner. All determinations of radioactivity were carried out in a gas flow windowless counting system, and the results are corrected for background, and in the case of carbonate, for self absorption.

Two types of experiments were carried out. In the 1st, the aim was to determine the relative effects on $O_2$-uptake of a group of phenols and other compounds, by testing responses over a wide range of concentrations. For such experiments 650 mg samples of discs were placed in Warburg flasks (with the test compound where necessary) in 2 ml 0.03 M potassium phosphate pH 5.0. $O_2$ uptake was measured over a 2-hour period in the usual manner at 25° C.

The information from these experiments was used to choose, for each compound, concentrations which were: a) slightly stimulatory, b) strongly stimulatory, and c) inhibitory to $O_2$-uptake. In the 2nd series of experiments the test compounds were supplied at each of these 3 levels and stimultaneous measurements of $O_2$-uptake and glucose uptake were made over a longer experimental period (6 to 8 hr). In these experiments 2 series of flasks were assembled. The 1st was composed of large vessels (100 ml capacity) and these contained 1.30 g discs, the test compound, and an aliquot of radioactive glucose (0.036 mg) (tipped from the side arm after equilibration) in 4 ml

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0.03 M phosphate pH 5.0. In order to measure glucose uptake, samples (0.1 ml, containing initially about 400 cpm) were removed at hourly intervals from these large flasks. These were dried under an infrared lamp on steel planchets and counted directly. The decrease in radioactivity was used to calculate glucose uptake.

The 2nd, parallel, series was made up of small flasks each containing 650 mg discs in 2 ml of solution with proportionate amounts of glucose and the test compounds. These flasks were used to obtain a continuous record of $O_2$-uptake of controls and treated samples during the experiment; they were not opened when samples were removed from the larger flasks. The respired $CO_2$ was trapped in 20 % sodium hydroxide and the radioactivity determined at the end of the experiment by converting to barium carbonate, plating and counting. For each of the compounds tested an experiment of this kind was carried out and then repeated at least 3 times. The results were then pooled and averaged. In the experiments with mitochondria, castor beans were used; the methods were those described by Beevers and Walker (3).

## Results

A. Experiments in Which Respiration Only Was Measured: When different concentrations of 2,4-dinitrophenol were applied, the anticipated stimulatory and inhibitory effects were established from the outset, and $O_2$-uptake continued unchanged at these new levels throughout the period of measurement. From data obtained in this way dosage response curves were constructed for each compound.

The forms of the various response curves were similar; for each compound only a rather narrow range of concentrations induced stimulated $O_2$-uptake, and inhibitory effects ensued when higher levels were applied. The response curves differed from each other in 2 respects; the extent of the stimulation induced at the most effective levels of the test compound and in the placing of the response curve along the concentration axis.

We may note from table I that on both of these counts unsubstituted phenol is the least effective compound; not only is the maximum stimulation a mere 20 % above the control, but the concentration required to induce this is considerably greater than 0.01 M. By contrast, dinitrophenol, the most effective compound, elicited a maximum response (236 % of the control rate) at a concentration 0.000025 M (table I).

Between these extremes lie the responses to the other compounds tested. The single substitution of Cl-, Br-, or $NO_2$-, in any position results in a compound greatly superior to phenol itself in its stimulatory ability. *Para*-substitution was particularly effective in all 3 classes, and resulted in stimulation as striking as those from dinitrophenol and dichlorophenol. However, the concentrations which were required to induce these effects were somewhat higher than the corresponding dinitrophenol level. These differences are shown in table I, in which the effectiveness of each compound in inducing stimulated respiration is related to that of dinitrophenol by the calculation of a DNP index.

For comparison, results obtained with azide and with methylene blue are included. In no case was azide found to induce a significant stimulation of respiration, methylene blue was effective over a rather wide range at high concentrations.

B. Experiments in Which Glucose Uptake and $O_2$-uptake Were Measured: The experimental design outlined above allowed the following

Table I

Effects of Various Compounds on the Oxygen Uptake of Carrot Discs

| Compound | Maximum stimulation observed | Concentration inducing maximum stimulation | Concentration inducing 50 % inhibition | DNP index * |
|---|---|---|---|---|
| | *% of Controls* | $M \times 10^{-5}$ | $M \times 10^{-3}$ | |
| Phenol | 120 | 2500 | 6600 | 1000 |
| *o*-Nitrophenol | 155 | 250 | 1000 | 100 |
| *m*-Nitrophenol | 227 | 250 | 580 | 100 |
| *p*-Nitrophenol | 242 | 60 | 480 | 25 |
| 2,4-Dinitrophenol | 236 | 2.5 | 20 | 1 |
| *o*-Chlorophenol | 180 | 760 | 1400 | 301 |
| *m*-Chlorophenol | 160 | 125 | 680 | 50 |
| *p*-Chlorophenol | 241 | 125 | 680 | 50 |
| 2,4-Dichlorophenol | 253 | 60 | 160 | 25 |
| *o*-Bromophenol | 200 | 76 | 630 | 30 |
| *m*-Bromophenol | 146 | 76 | 400 | 30 |
| *p*-Bromophenol | 232 | 76 | 400 | 30 |
| Methylene blue | 170 | 50 | 1600 | 20 |
| Sodium azide | No stimulation from $10^{-8}$ M—3 $\times$ $10^{-5}$ M | | 5 | ... |

$$\text{* DNP index} = \frac{\text{Molar concentration of test compound giving maximum stimulation}}{\text{Molar concentration of 2,4-dinitrophenol giving maximum stimulation}}$$

determinations to be made: a) a continuous record of $O_2$-uptake b) hourly progress of glucose uptake and c) total $C^{14}O_2$ release, determined at the end of the experiment. From this primary data the information contained in table II was obtained. The effects of the test compounds on $O_2$-uptake, glucose uptake, and $C^{14}O_2$ output are recorded as percentages (table II) of the corresponding control rates. As indicated at the foot of table II the control values observed in the several experiments are quite uniform. They show that the bulk of the supplied glucose was absorbed during each experiment and that only a small fraction of this was converted to $C^{14}O_2$. The responses to the various test compounds at each of the 3 chosen concentrations are shown in table II. Again a uniform pattern emerges.

The $O_2$-uptake figures show firstly that the anticipated respiratory responses were induced, although it should be noticed that the stimulations are not, in general, so large as those observed in the corresponding short term experiments. Strong inhibitions of glucose uptake are induced by concentrations of the phenols which curtail $O_2$-uptake, but what is more important, striking inhibitions are obtained at those levels which are most effective in stimulating respiration, and correspondingly smaller effects, again inhibitory ones, are obtained at still lower levels of the test compounds, which elicit smaller respiratory stimulations. The values in table III emphasize one important aspect of the results, that all of the test compounds, at levels which reduced glucose uptake by half, induced stimulations in the rate of $O_2$-uptake (table III).

For each substituted phenol the curves relating its concentration to its effects on glucose uptake and $O_2$-uptake, diverged in opposite directions from the horizontal when a particular concentration was exceeded. Thus in their relative effectiveness as inhibitors of glucose uptake the various phenols are related to dinitrophenol in the same way as they were found to be in the respiration experiments. That is to say, dichlorophenol, *p*-nitrophenol, and the *m*- and *p*-substituted chloro- and bromo-phenols were 20 to 50 times less potent than dinitrophenol, and the *o*-substituted phenols were the least effective.

It will be seen that in a few instances (*m*- and *p*-substituted nitro- and chloro-phenols) in table II, glucose uptake was apparently less strongly inhibited at the highest concentration of phenol than it was at the intermediate level. It was noticed that in those examples a considerable amount of "leakage" had occurred from the slices during the experiment so that on drying down the aliquots removed for counting a lacquer-like deposit was obtained, whereas normally only a very thin film was obtained on drying. The

TABLE II

SIMULTANEOUS OBSERVATIONS ON THE UPTAKE OF OXYGEN AND RADIOACTIVE GLUCOSE BY CARROT DISCS IN RESPONSE TO SUBSTITUTED PHENOLS

| COMPOUND | LOG. MOLAR CONCENTRATION | OXYGEN UPTAKE | SUGAR UPTAKE | $C^{14}O_2$ EVOLVED |
|---|---|---|---|---|
| | | *% of controls* * | | |
| *o*-Nitrophenol | −3.2 | 108 | 89 | 84 |
| | −2.6 | 130 | 26 | 49 |
| | −2.2 | 70 | 3 | 5 |
| *m*-Nitrophenol | −3.3 | 110 | 84 | 101 |
| | −2.6 | 117 | 6 | 3 |
| | −2.2 | 21 | 18 | 2 |
| *p*-Nitrophenol | −3.3 | 168 | 36 | 47 |
| | −3.1 | 91 | 14 | 16 |
| | −2.3 | 6 | 37 | 1 |
| 2,4-Dinitrophenol | −5.6 | 160 | 88 | 96 |
| | −4.6 | 220 | 44 | 51 |
| | −3.6 | 14 | 17 | 2 |
| *o*-Chlorophenol | −3.2 | 116 | 58 | 61 |
| | −2.9 | 128 | 35 | 27 |
| | −2.6 | 144 | 26 | 9 |
| *m*-Chlorophenol | −3.2 | 113 | 30 | 31 |
| | −2.9 | 137 | 15 | 9 |
| | −2.3 | 73 | 25 | 1 |
| *p*-Chlorophenol | −3.4 | 181 | 39 | 63 |
| | −2.9 | 185 | 6 | 7 |
| | −2.2 | 4 | 35 | 1 |
| 2,4-Dichlorophenol | −4.3 | 123 | 86 | 81 |
| | −3.2 | 195 | 10 | 9 |
| | −2.9 | 37 | 2 | 3 |
| *o*-Bromophenol | −4.3 | 110 | 96 | 89 |
| | −3.3 | 117 | 76 | 86 |
| | −2.3 | 110 | 18 | 6 |
| *m*-Bromophenol | −4.3 | 106 | 100 | 101 |
| | −3.1 | 115 | 25 | 19 |
| | −2.6 | 71 | 22 | 25 |
| *p*-Bromophenol | −4.3 | 107 | 96 | 93 |
| | −3.3 | 150 | 35 | 53 |
| | −2.6 | 47 | 17 | 1 |

* The control samples in the above experiments took up from 76 to 93 % (average 84 %) of the supplied glucose-$C^{14}$ and converted 8.1 to 11.9 % (average (10.0 %)) of this to $C^{14}O_2$.

TABLE III

RESPIRATORY RESPONSES TO SUBSTITUTED PHENOLS IN CONCENTRATIONS WHICH INHIBITED GLUCOSE UPTAKE BY 50 %

| COMPOUND | CONCENTRATION AT WHICH GLUCOSE UPTAKE WAS INHIBITED BY 50 %. $M \times 10^{-5}$ | RESPIRATION AT THIS CONCENTRATION ($O_2$-UPTAKE AS % OF CONTROLS) |
|---|---|---|
| *o*-Nitrophenol | 159 | 123 |
| *m*-Nitrophenol | 87 | 116 |
| *p*-Nitrophenol | 42 | 167 |
| 2,4-Dinitrophenol | 2 | 218 |
| *o*-Chlorophenol | 79 | 120 |
| *m*-Chlorophenol | 25 | 105 |
| *p*-Chlorophenol | 23 | 170 |
| 2,4-Dichlorophenol | 19 | 158 |
| *o*-Bromophenol | 178 | 113 |
| *m*-Bromophenol | 32 | 115 |
| *p*-Bromophenol | 27 | 138 |

effect of the materials lost from the slices would be to increase self absorption and so to give a lower counting rate. This would lead to a spuriously high value for glucose uptake. We do not feel justified therefore in attaching significance to the apparently lower effectiveness of the highest concentrations of some of the test compounds.

The figures for $C^{14}O_2$ output during the experiments with glucose-$C^{14}$ are shown in the final column of table II. It should be emphasized that, of the total glucose absorbed by the controls, only about 10 % was converted to $CO_2$. Nevertheless it is clear that the test compounds drastically reduced the production of $C^{14}O_2$ at whatever level they were applied. The very close parallel between the responses of $C^{14}O_2$ production and glucose uptake to individual concentrations of the test compound should be especially noted.

C. Experiments with Mitochondria: The responses of plant mitochondria to DNP have been described by several authors (1, 6, 16, 17, 18) and it is clear that P/O ratios are drastically reduced. These experiments have usually been carried out in the presence of the hexokinase-glucose trapping system, which, although it allows the inhibitory effect of DNP on phosphate esterification to be shown, may preclude the demonstration of what is an equally distinctive effect, namely that of stimulated $O_2$-uptake. In castor bean mitochondria such a stimulatory effect of DNP has been described, (1), and we have now investigated the ability of other substituted phenols to bring about this response. For each compound a series of concentrations was applied and response curves were constructed from the results of triplicate experiments. The values in table IV were read off from the curves (table IV). Striking stimulations were induced by all of the phenols; again the disubstituted phenols were particularly effective, with the *p*-, *m*-, and *o*-substituted phenols following in that order. Methylene blue induced a considerable stimulation while azide had virtually no such effect. Inhibitions of $O_2$-uptake ensued when the concentrations of all the compounds were raised above the stimulating one.

Table IV

Effects of Various Substituted Phenols, Sodium Azide and Methylene Blue on the Oxygen Consumption by Mitochondria from Castor Bean Endosperm *

| Test compound | Maximum stimulation | Molar conc giving maximum stimulation | Molar conc giving 50 % inhibition |
|---|---|---|---|
| | % *of control* | $M \times 10^{-3}$ | $M \times 10^{-3}$ |
| *o*-Nitrophenol | 184 | 5.0 | 9.8 |
| *m*-Nitrophenol | 205 | 1.3 | 5.5 |
| *p*-Nitrophenol | 204 | 0.4 | 5.3 |
| Dinitrophenol | 204 | 0.3 | 6.3 |
| *o*-Chlorophenol | 146 | 3.7 | 12.0 |
| *m*-Chlorophenol | 141 | 2.5 | 4.9 |
| *p*-Chlorophenol | 189 | 2.5 | 7.6 |
| Dichlorophenol | 182 | 0.5 | 2.8 |
| Sodium azide | 107 | 0.5 | 3.1 |
| Methylene blue | 145 | 0.3 | 47.9 |

* The mitochondria were prepared as described by Beevers and Walker and $O_2$-uptake was measured over a 30-minute period with α-ketoglutarate (0.005 M) as substrate (3).

## Discussion

It is clear from the results that all of the substituted phenols, when applied at appropriate concentrations have the following properties in common with the classical uncoupling agent DNP: a) They stimulate the respiration of carrot discs; b) at levels stimulatory to $O_2$-uptake, they inhibit the associated endergonic process of glucose uptake; and c) they stimulate the $O_2$-uptake of castor bean mitochondria oxidizing α-ketoglutarate.

It is concluded that such compounds, like DNP, are uncoupling agents.

From their different abilities to induce the responses described (tables I, II and IV), the following conclusions were drawn about the relative effectiveness of the various phenols. 2,4-dinitrophenol was the most effective compound, i.e., it induced responses at the lowest levels. This was followed in turn by 2,4-dichlorophenol, and the *para*-substituted nitro-, chloro- and bromo-phenols; the *ortho*-substituted compounds were less effective but considerably more active than phenol itself. The responses to the addition of various substituents in the molecule thus conform in general to a pattern which has emerged from more extensive comparative work with other materials, in which usually only one response, i.e., a respiratory effect or an inhibitory action, has been measured. Among this work may be mentioned particularly that of Stenlid on wheat roots (24), Clowes and Krahl (7, 14) on sea urchin eggs, of Simon and Blackman (20, 22) on *Trichoderma viride*, *Lemna minor* and *Brassica alba* and of Field, Martin and Field (9) and more recently Deken (8) on yeast. We suppose, with the previous authors, that the differences in effectiveness most probably reflect, to a large degree, the ease of penetration of the phenols to the points of primary attack, which, one would now suppose, are the enzymes in the mitochondria concerned with electron transfer.

Azide failed to induce respiratory stimulations in the experiments reported here, although effects which can be reasonably ascribed to an uncoupling action have been described in plants by Stenlid (23) and by Harley and his colleagues (12). Presumably respiratory stimulations can only be demonstrated if the level of azide required is lower than that at which the oxidation enzymes are themselves inhibited. As in Stenlid's experiments (25) methylene blue induced effects on respiration and glucose uptake which were similar to those due to the phenols. This parallelism has now been shown to extend to the mitochondria. It should be noticed however, that the mechanism of action of methylene blue may be rather different from that of the phenols, since it is itself autooxidizable

and may effectively by-pass normal electron transfer steps (13).

In considering the $C^{14}O_2$ results it should first be emphasized that the carrot tissue contains ample available carbohydrate reserves, and its respiration, with a respiratory quotient close to unity is not increased by the small amounts of glucose supplied. At all levels of DNP (and, by inference, of the other phenols) which stimulate $O_2$-uptake, $CO_2$-output is stimulated to at least the same extent (2). But, at these stimulatory levels, as shown in table III $C^{14}O_2$ output was drastically reduced, so that the specific activity of the respired $CO_2$ declines with increasing DNP. Such an observation was one of the arguments used by Porter and Runeckles (19) in support of an otherwise well founded suggestion that in tobacco leaves DNP induced the respiration of non-carbohydrate material. However, the final 2 columns in table II show that there is a striking parallelism between the inhibitory effects of each phenol on glucose uptake and on $C^{14}O_2$ release. In the carrot tissue then, it is possible to ascribe the lowered specific activity of the $CO_2$ directly to a reduced uptake of the radioactive glucose, and it is not necessary to invoke a change in respiratory substrate as an explanation. It should be noted that in contrast to the carrot tissue used in these experiments and those described previously (2) the tobacco leaf tissue had a low RQ (0.7), its respiration was strikingly stimulated by the added sugar, and the experiments were carried out in 100 % $O_2$. Whatever may be the explanation of the responses to DNP in the tobacco tissue, the present results confirm that no change is necessary in the conclusions from our previous experiments, (2, 10), namely that the respiratory responses to DNP in the tissues used can be ascribed to an increased rate of glycolysis brought about as an indirect result of the uncoupling action at the electron transfer level.

## Summary

The effects of a series of substituted phenols on $O_2$-uptake and glucose accumulation by carrot discs have been determined. The response to each compound was a respiratory stimulation, which reached a maximum and then declined sharply to an inhibition as the concentration was raised over a rather narrow range. The striking changes in respiration as the concentrations were increased were accompanied by the progressive inhibition of glucose absorption; those concentrations which induced maximally stimulated $O_2$-uptake drastically curtailed glucose uptake. The various phenols induced stimulations of $O_2$-uptake by castor bean mitochondria which were similar to those induced by dinitrophenol. The various substituted phenols were thus supposed to be acting, with varying degrees of efficiency, as uncoupling agents.

The relative effectiveness was gauged by comparing the concentrations of the compounds required to induce maximally stimulated respiration, which in most instances amounted to over 200 % of the control rates. 2,4-Dinitrophenol was the most effective compound, followed, in turn by 2,4-dichlorophenol, *p*-nitrophenol, the bromophenols, *m*- and *p*-chlorophenol, *m*- and *o*-nitrophenol, *o*-chlorophenol and phenol itself, which induced at best only 20 % stimulation.

In parallel experiments with methylene blue it was shown that effects similar to those of the substituted phenols were induced. Azide induced only respiratory inhibitions and corresponding curtailment of glucose accumulation in carrot discs.

It was also demonstrated that the appearance of radioactive carbon in the respired $CO_2$ was inhibited only to the same degree as the uptake of the glucose-$C^{14}$ was itself prevented. For this reason the effects of substituted phenols on $C^{14}O_2$ production cannot be considered as evidence for a changeover in respiratory substrate in response to their addition.

This work has been supported in part by the Atomic Energy Commission, (Contract no. AT-(11-1)-330). We record our appreciation of this support.

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# STUDIES ON THE MECHANISM OF HERBICIDAL ACTION OF 2-CHLORO-4,6-BIS(ETHYLAMINO)-S-TRIAZINE [1, 2]

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Recent studies have shown that certain triazine derivatives can be used to control weeds in specific crops at relatively low rates of application (2, 3). At higher rates of application they can be used as soil sterilants for general weed control on non-cropped areas. One of these compounds, 2-chloro-4,6-bis-(ethylamino)-s-triazine [simazin] [5], applied as a pre-emergent spray at 2 to 4 pounds per acre has given excellent control of annual weeds in corn for practically the entire growing season (3). Corn appears to be especially tolerant to the chemical and very high rates of application have been used successfully in some studies. Barley and other small grains are not able to tolerate pre-emergent applications of even 2 pounds per acre of simazin (2, 3).

The objectives of the studies reported herein were a) to determine the effects of simazin on the photochemical activity of chloroplasts isolated from barley plants and b) to determine whether the inhibitory effects of simazin could be overcome by supplying treated barley plants with carbohydrates from an external source. A preliminary report (4) of these findings has been presented.

[1] Received November 24, 1958.
[2] Cooperative investigations of the Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture and the North Carolina Agricultural Experiment Station. Published with the approval of the North Carolina Agricultural Experiment Station as Paper no. 933.
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## MATERIALS AND METHODS

STUDIES WITH ISOLATED CHLOROPLASTS: The chloroplasts used in this study were obtained from 10-day-old greenhouse-grown barley plants (*Hordeum vulgare* L. variety Dayton, CI 9517) using the procedure given by Spikes et al (6). Following the initial isolation they were washed 3 times with 0.5 M sucrose.

The photochemical activity of the isolated chloroplasts was measured in small beakers. A 10-ml reaction mixture was used which had the following composition: 0.0005 M potassium ferricyanide, 0.01 M potassium chloride, 0.17 M sucrose, 0.05 M potassium

phosphate buffer (pH 6.85), 2 % ethanol by volume, and an aliquot of chloroplasts that contained 0.3 mg of chlorophyll. Prior to use in the reaction the suspension of chloroplasts was maintained in an ice bath in the dark. Chlorophyll concentration was determined following the procedure of Arnon (1). Tank nitrogen, after being passed through alkaline pyrogallol, was continuously bubbled through the reaction mixture to keep the chloroplasts stirred and in suspension. The reaction vessels were illuminated from below with a 150-watt reflector-type floodlight bulb. To trap the heat emitted by the lamp a water filter was interposed between it and the reaction vessel. Running tap water was passed through this filter. With this arrangement, the temperature of the reaction mixture varied less than 1° C during the illumination period. The lower surface of the chloroplast suspension was illuminated at an intensity of approximately 2,800 ft-c. Under the conditions established for this study maximum reduction was attained at an intensity of approximately 750 ft-c. Consequently, light should not have been limiting the rate of the reaction.

The activity of the chloroplasts was followed potentiometrically at room temperature (ca. 26° C) with platinum and saturated calomel electrodes used in conjunction with a Beckman model H-2 pH meter. In making measurements, the system without the chloroplasts was allowed to equilibrate in the dark for 2 minutes. The aliquot of chloroplasts was subsequently added and the light turned on. Millivolt readings were recorded over a 4-minute period. These values were converted to micromoles of ferricyanide reduced during the course of the experiment by use of the relationship given by Spikes et al (6).

Since simazin is relatively insoluble in water, solutions of the desired strength were prepared with absolute ethanol. The final concentration of ethanol in all the reaction mixtures was held constant at 2 % by volume. This concentration of ethanol had no detectable effect on the reaction.

Studies with Intact Barley Plants: Barley plants were grown under greenhouse conditions in polyethylene cups, 7-cm in diameter at the top, containing perlite saturated with Hoagland's solution. Ten days after seeding, when the plants were 10- to 12-cm tall and in the 2-leaf stage, the herbicide was dispersed in 20-ml of tap water and applied to the surface of the perlite. (A 50 % wettable powder formulation was used.) In preliminary experiments, 0.75 mg/cup (the equivalent of 1 lb. active ingredient/acre) was found to be a concentration which consistently killed the plants in a 10-day period. Rates up to 3.0 mg/cup (4 lb./acre) did not kill the plants more rapidly.

Immediately following the application of the herbicide, the treated plants were divided into 3 groups of 5 plants each. The terminal 5-mm was removed from both leaves of all plants. Plants in group 1 received no additional treatment. Leaves of plants in groups 2 and 3 were inserted in small vials. The vials contained distilled water and 4-ml of an autoclaved 6 % glucose solution, respectively. The leaves were held in position in the supported vials with cotton plugs. Fresh cuts were made at the tips of the leaves every 2nd day. At this time the solutions in the vials were changed and 20-ml of water was added to the perlite in each container. Control plants were handled in a similar manner. These experiments were replicated 5 times over a 7-month period.

## Results and Discussion

Studies with Isolated Chloroplasts: Simazin, at low concentrations, was found to inhibit the photochemical activity of isolated chloroplasts. The inhibition obtained with different concentrations of simazin is shown in figure 1. These data were obtained by comparing the micromoles of ferricyanide reduced over a 4-minute period in the presence of various concentrations of simazin with the amount reduced under identical conditions in the absence of the compound. The decrease in activity is expressed on a percentage basis and is plotted as a function of the log of the molar concentration of the inhibitor.

Data used to obtain the point on the curve for the $2.5 \times 10^{-6}$ M level of simazin are presented in table I. Shown in the table are a) the magnitude of ferricyanide reduction obtained under the conditions of this investigation, b) the corrections made for reduction which occurred in the dark, and c) the method used to calculate percent inhibition. The reduction that occurred in the dark was apparently produced non-enzymatically since it was also obtained with chloroplast preparations which had been boiled

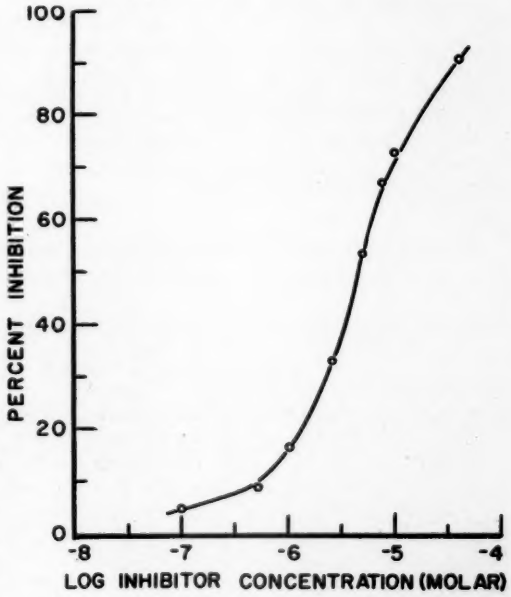


Fig. 1. Simazin inhibition of ferricyanide reduction (Hill reaction) by isolated chloroplasts of barley.

TABLE I

EFFECT OF 2.5 × $10^{-6}$ M SIMAZIN ON THE PHOTOCHEMICAL ACTIVITY OF ISOLATED BARLEY CHLOROPLASTS

| REPLICATE SERIES | TREATMENT | REDUCTION OF FERRICYANIDE | | | INHIBITION* |
|---|---|---|---|---|---|
| | | LIGHT (L) | DARK (D) | NET (R=L−D) | |
| | | μM | μM | μM | % |
| 1 | Control | 2.835 | 0.085 | 2.750 | |
| | Simazin | 1.889 | 0.084 | 1.805 | 34 |
| 2 | Control | 3.067 | 0.076 | 2.991 | |
| | Simazin | 2.115 | 0.069 | 2.046 | 32 |
| 3 | Control | 3.067 | 0.076 | 2.991 | |
| | Simazin | 2.068 | 0.089 | 1.979 | 34 |
| 4 | Control | 2.268 | 0.063 | 2.205 | |
| | Simazin | 1.528 | 0.074 | 1.454 | 34 |
| 5 | Control | 2.153 | 0.065 | 2.088 | |
| | Simazin | 1.487 | 0.064 | 1.423 | 32 |
| | | | | Average | 33 |

* Percent inhibition $= \frac{R_{control} - R_{simazin}}{R_{control}} \times 100.$

for 5 minutes. Values for the other points on the curve were obtained in the same manner. Determinations were made on 5 different days over a 17-day period with chloroplasts from 3 different plantings of barley. Control determinations were made at various intervals throughout each replication. The individual control values were averaged together to obtain a single value for each replication which was used in making the calculations. In the first replicate, 4 control runs were involved while 5 control runs were made in the other replicates.

The concentration of simazin required to reduce the photochemical activity of barley chloroplasts by 50 % ($I_{50}$) was 4.6 × $10^{-6}$ M. These data suggest that interference with photosynthesis may be one of the mechanisms through which this chemical controls plant growth. If photosynthesis is the major mechanism involved in the phytotoxic action of simazin, it should be possible to circumvent the action of the herbicide by supplying carbohydrates from an external source to simazin-treated plants. This hypothesis was subsequently tested.

STUDIES WITH INTACT BARLEY PLANTS: During the 1st 7 days following the application of 0.75 mg (1 lb./acre) of simazin, plants that received no glucose developed necrotic areas and subsequently became flaccid. By the 10th day the plants appeared to be completely desiccated. The necrotic symptoms on the plants which had their leaves immersed in distilled water were decreased in intensity but appeared later. No new growth, as evidenced by a failure of the 3rd leaf to develop, was observed in either of these 2 groups of plants. Symptoms of injury could not be detected on plants which had been supplied glucose through their leaves from an external source. The protective action of the glucose solution in overcoming the inhibiting effect of simazin on the development of the 3rd leaf is apparent in figure 2.

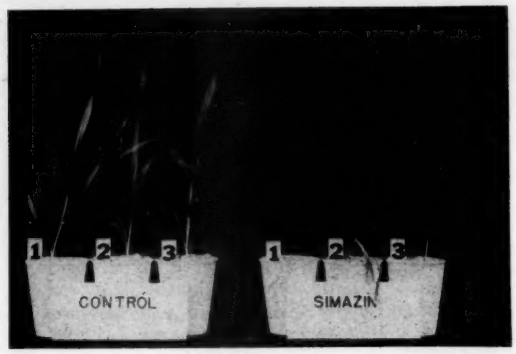


FIG. 2. Glucose protection of barley seedlings during a 10-day growth period against the lethal effect of simazin. The group of plants at the left (control) received no simazin. Those on the right received 0.75 mg/cup (1 lb./acre) at the 2-leaf stage of growth. Tips of the 1st 2 leaves of all plants were clipped and 1) not immersed, 2) immersed in water, and 3) immersed in a 6 % glucose solution.

Data which show the effect of the different leaf treatments on the length of the 3rd leaf and on the fresh weights of shoots and roots are presented in table II. These data were obtained 10 days following treatment with the herbicide. Inhibition was calculated on a percentage basis by comparing herbicide-treated plants with control plants. By introducing glucose the lethal action of simazin was overcome

TABLE II

MODIFICATION OF SIMAZIN TOXICITY TO BARLEY BY GLUCOSE

| LEAF TREATMENT | SIMAZIN PER CUP | THIRD LEAF | | TOPS | | ROOTS | |
|---|---|---|---|---|---|---|---|
| | | LENGTH* | INHIBITION | FRESH WEIGHT* | INHIBITION | FRESH WEIGHT* | INHIBITION |
| | *mg* | *cm* | % | *g* | % | *g* | % |
| None | ... | 32 | ... | 1.06 | ... | 1.14 | ... |
| None | 0.75 | 3 | 91 | 0.10 | 91 | 0.12 | 90 |
| Water | 0.75 | 3 | 91 | 0.24 | 77 | 0.18 | 84 |
| 6 % glucose | 0.75 | 22 | 31 | 0.70 | 34 | 0.38 | 67 |

* Average of 5 plants.

and its effect on growth was partially offset. This circumventing effect on growth was more pronounced in the shoots than in the roots. Water supplied through the leaves of simazin-treated plants prevented desiccation. The slight increase in fresh weight resulting from this treatment is not considered to represent new growth. No additional growth of new leaves was ever observed on plants that received only water through their leaves.

Development of the 4th leaf was delayed even on the glucose-treated plants. This is evident in figure 2 and is reflected in the data presented in table II. The inability to obtain complete protection from the effects of simazin may be due to an inadequate supply and/or inefficient translocation of the glucose to the various developmental tissues of the plant. However, inhibition by the herbicide of essential metabolic processes in addition to photosynthesis must also be considered a definite possibility.

In other experiments, barley plants to which carbohydrates were made available from an external source have been kept alive for more than 2 months. During this time, 8 or 9 leaves developed even in the presence of concentrations of simazin as high as 3 mg/cup(4 lb./acre).

In view of the relatively high tolerance of corn to simazin, some preliminary studies have been made with chloroplasts isolated from young greenhouse-grown plants. The activity of chloroplasts from both barley and corn appear to respond similarly to simazin. Consequently, the mechanisms that control selectivity must be assumed to act before the herbicide reaches the chloroplasts. These mechanisms, however, remain to be characterized. A study on this problem has been reported by Roth (5). He found that corn extracts contained a thermolabile component which could alter the structure of the simazin molecule. Extracts obtained from wheat, a species which is not as tolerant to simazin as corn, lacked this constituent.

The ability of glucose to keep barley plants alive and growing in the presence of lethal concentrations of simazin supports the hypothesis that this chemical may kill the plant by interfering with the photosynthetic mechanism. The extreme sensitivity of the photochemical activity of the chloroplasts to simazin suggests that a major site of action may be that which is concerned with the photolysis of water.

[During the final preparatory stages of this manuscript, a brief report came to our attention in which inhibition of the Hill reaction by simazin had been independently observed (B. Exer, Experientia 14: 136–137. 1958)].

## Summary

Simazin reduced the photochemical activity (Hill reaction) of isolated barley chloroplasts by 50 % at $4.6 \times 10^{-6}$ M. Glucose supplied to barley plants through severed leaf tips kept plants alive and growing in the presence of lethal concentrations of simazin.

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# COMPARISON OF THE NUCLEIC ACID, TOTAL NITROGEN, AND PROTEIN NITROGEN LEVELS OF NORMAL AND TUMOR TISSUE UNDER SIMILAR GROWTH CONDITIONS [1, 2]

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Many studies have been made of the differences and similarities of normal and crown gall tissues. These studies have been the subject of a number of excellent reviews (8), and no attempt will be made to review the literature here. Klein (7) has made an extensive study of the differences between normal and tumor tissue with respect to nitrogen and phosphorus content and respiratory rates. In this work the comparison was made between tumors induced in a plant and the normal tissue of the host species. In spite of the value of such studies in determining the status of crown gall metabolism in vivo, they are open to the criticism that differences in the growth rates and differences in the degree of differentiation of the host and tumor cells may obscure the true picture of the metabolic changes brought about by tumor

[1] Received revised manuscript November 24, 1958.
[2] This work was supported in part by a grant, no. C-2154, from the National Institutes of Health; and a grant from the Smith, Klein and French Foundation.

induction. These difficulties can be overcome by the use of tissue cultures. In vitro culture avoids host-tumor competition and interaction, permits maximal control of the tissue environment, and makes possible the selection of normal and tumor tissue of essentially undifferentiated nature. The senior author has developed such cultures during the past 5 years and has used these to make comparative studies of the growth rate, alkaloid conversions, and effects of pH and inhibitors on the respiration of normal and tumor tissue (these data will appear elsewhere (15)).

Several investigators have used tissue culture techniques to investigate the differences in normal and crown gall tissue. It has been shown that there is a specific protein fraction peculiar to the Parthenocissus tumor tissue (2), and that there are quantitative differences in phosphorus and nitrogen content (10) as well as respiratory rate and enzymatic activity (4) of grape tissues and salsify tissue (9). However, no investigation has been made of the possible genetic differences between these tissues. Since the induction of the tumor involves a drastic change in DNA content (8), it is of interest to know whether the 2 types of tissues can be distinguished on the basis of a change in nucleic acid levels. Any transformation of this type would be expected to have a profound influence on the total metabolism of the tissue.

Furthermore, no studies have been made to show the effect of growth rate on the constitution of these cells. The problem is this: are the differences between normal and tumor tissue the result of intrinsic alteration of the entire metabolism, or are they merely by-products resulting from the alteration of a single growth-rate controlling step? The profound difference in the auxin requirements of the 2 tissues and the relation between auxin and nucleic acid levels (18) suggest the possibility of the latter. Therefore, it seemed worthwhile to assay these 2 tissues under similar conditions of growth, and under conditions in which some interference with the auxin metabolism altered the growth rate of the tissues.

Recently, studies have been completed on the effects of 7-azaindole propionic acid on the growth of tissue cultures (16). These studies indicated the possibility of using this azaauxin as a growth regulator for studies of comparative metabolism. The data presented in this paper are the results of preliminary studies on the general problem outlined above, using the azaauxin as a tool to alter the growth rates of both normal and tumor tissue.

## Materials and Methods

Normal and bacteria-free crown gall tissues of *Parthenocissus tricuspidata* were used in all experiments. The tissue did not contain chlorophyll. Other changes may have occurred in the tissue. The normal tissue is referred to as "normal" hereafter only to distinguish it as being derived from normal stem tissue as opposed to crown gall tissue and also to distinguish it from habituated tissue which is able to grow in the absence of external auxin supply.

The cultures were grown at 29° C on agar slants containing a modification of White's medium (19). The culture techniques are described elsewhere (16). All cultures were grown in the presence of $\beta$-(7-aza-3-indoyl)-propionic acid (14), referred to as "aza-auxin" throughout the text. The normal tissue was grown on either 0.10 mg/l (0.05 $\mu$M/l) or 0.40 mg/l; the tumor tissue was grown on either 0.10 mg/l or 0.50 mg/l. (Naphthaleneacetic acid, which is normally used to supply the auxin requirement of the normal tissues, was omitted in all cultures.) For simplicity these tissues are designated N-1, N-4, T-1, T-5 throughout the text. The N-4 concentration of azaauxin was used for the normal tissue as the tissue showed no growth at the N-5 concentration. This is an indication of the greater resistance to inhibition shown by the tumor tissue (16).

The cultures were harvested after 3 weeks, weighed, and then dried at 65° C. The dry weights were determined and the tissue stored in a dessicator until assay. For assay the tissue was ground and then weighed. For extraction of nucleic acids and for phosphorus fraction assay, 0.1 to 0.2 g of dried tissue were used. For total nitrogen analysis, 0.1 g, and for protein nitrogen, 0.2 g of tissue were used.

The nucleic acid and the prosphorus fractions were obtained by the method of Ogur and Rosen (11). There were 5 main fractions analyzed: alcohol soluble fraction (I), alcohol-ether soluble fraction (II), acid soluble fraction (III), RNA fraction, and DNA fraction. The nucleic acid fractions were analyzed by use of their absorption at 2600 Å in the Beckman spectrophotometer, and by analysis of the phosphorus content. Phosphorus was estimated by the method of Fiske and SubbaRow (5). The amount of DNA was also determined by the colorimetric method (diphenylamine reaction) in a few cases.

The total and protein nitrogen were assayed by use of the semimicro Kjeldahl-Gunning-Jodlbauer method (1), using Henger granules and copper as catalysts. In the protein nitrogen assays, the tissue was subjected to a continuous 18-hour extraction with 75% alcohol followed by a 20-minute water extraction in a Soxhlet apparatus before Kjeldahl digestion. Thus the "protein nitrogen" represents hot alcohol and hot water insoluble nitrogen. The nitrogen released by digestion was determined by titration.

Cell counts were made after removal of the intercellular matrix with a nitric acid-chromic acid macerating fluid (3). One tenth ml of a diluted preparation was put into a depression slide and the cells counted. Several determinations of each digestion were made and several digestions of similar types of tissue performed to give the average cell number used in this paper. It is interesting to note that the cell number is very constant for each type of tissue used under the conditions of these studies. Other studies suggest that the cell number of normal tissue may vary with varying conditions.

Chromosome determinations were made by use of Feulgen smears of the 2 types of tissue. Counts were made of several different cultures and of cul-

TABLE I

CHANGES IN NUCLEIC ACID LEVELS IN NORMAL AND CROWN GALL CULTURES *

| TISSUE ** | No. TESTS | RNA MG/G DRY WT | DNA MG/G DRY WT | RNA MG/$10^6$ CELLS | DNA MG/$10^6$ CELLS | RNA/DNA |
|---|---|---|---|---|---|---|
| N-1 | 4 | 3.61 | 2.69 | 0.082 | 0.061 | 1.34 |
| N-4 | 4 | 4.44 | 2.73 | 0.103 | 0.063 | 1.63 |
| T-1 | 4 | 3.09 | 2.47 | 0.182 | 0.145 | 1.25 |
| T-5 | 4 | 3.68 | 2.37 | 0.245 | 0.158 | 1.55 |

* Data from determination of RNAP and DNAP converted to RNA and DNA (17).

** N-1—44 $\times$ $10^6$ cells; N-4—43 $\times$ $10^6$ cells; T-1—17 $\times$ $10^6$ cells; T-4—15 $\times$ $10^6$ cells per gram dry weight. Average fresh weight per culture: N-1—0.204 g; N-4—0.087 g; T-1—0.524 g; T-5—0.253 g.

tures grown both in the presence and absence of colchicine. The pattern of cell division seems to be quite regular (16), with the majority of division figures appearing about midnight, 3 days after the introduction of the culture. Addition of 0.01 % colchicine to the medium resulted in an increased number of recovered division figures and more readily spread-out figures. Counts were made of the nucleolar number in stained and unstained cells (phase contrast). Estimations of nuclear volume were made by use of a Filar ocular micrometer; however, the irregular shapes of the nuclei of both types of tissue made these estimates very difficult.

## RESULTS

The changes in nucleic acid content of normal and tumor tissue which were induced by different concentrations of azaauxin are presented in tables I and II. On the basis of fresh and dry weights, the cultures N-4 and T-5 were growing at approximately one half the rate of cultures N-1 and T-1 (ratio of fresh weights T-5/T-1 0.48; N-4/N-1 0.47) (16). The data in table I were obtained by analysis of the phosphate content of the RNA and DNA by use of the conversion factor suggested by Schneider (17). The data in table II are derived from spectrophotometric studies. It can be seen that the estimate of the nucleic acid content of the tissues is higher by a factor of approximately 2 if the phosphate data are used. At the present time we cannot offer any satisfactory explanation for this observation. It appears that some phosphate containing entity is in this fraction in addition to the nucleic acids themselves. (This may represent a phospho-protein.) As a result, the phosphate data must be considered unreliable with regard to absolute amounts of nucleic acid present in the fractions. However, this reservation casts no doubt on the general conclusions which can be drawn from these data as the same relative trend is seen in both tables I and II; and this trend is borne out by determinations made with the colorimetric method (diphenylamine). In the colorimetric studies the same relationship holds for the nucleic acids.

It is evident, therefore, that on a per-cell basis the nucleic acid content of the tumor tissue is about 2.5 times that of the normal tissue under these conditions of growth. Lowering of the growth rate by higher concentrations of azaauxin causes a consistent, although small, increase in the nucleic acid content of both types of tissue. The percentage increase is the same in both the normal and tumor tissue. In no case does the value for the normal tissue approach that for the tumor as long as the per-cell basis is used.

The data in table I represent averages of 4 separate batches of tissue. The data in table II represent averages from 3 of these same batches and 4 others (7 total).

Table III presents the changes in total nitrogen and protein nitrogen content when the cultures are grown in the presence of different concentrations of azaauxin. If the per-cell figures are taken, it is apparent that once again the tumor tissue has about 2 times as much nitrogen as the normal tissue and once again the amount of nitrogen increases in both tissues as the rate of growth is slowed. However, the increase in RNA does not cause a concomitant increase

TABLE II

CHANGES IN NUCLEIC ACID LEVELS IN NORMAL AND CROWN GALL CULTURES *

| TISSUE ** | No. TESTS | RNA MG/G DRY WT | DNA MG/G DRY WT | RNA MG/$10^6$ CELLS | DNA MG/$10^6$ CELLS | RNA/DNA |
|---|---|---|---|---|---|---|
| N-1 | 7 | 1.47 | 1.42 | 0.033 | 0.032 | 1.03 |
| N-4 | 7 | 1.77 | 1.77 | 0.041 | 0.041 | 1.00 |
| T-1 | 7 | 1.51 | 1.31 | 0.089 | 0.077 | 1.16 |
| T-5 | 7 | 1.76 | 1.67 | 0.117 | 0.109 | 1.07 |

* Data from determination of RNA and DNA by spectrophotometric method.

** Cell numbers the same as table I.

TABLE III

CHANGES IN TOTAL NITROGEN AND PROTEIN NITROGEN IN NORMAL AND CROWN GALL TISSUES

| TISSUE* | No. TESTS | TOTAL NITROGEN MG/G DRY WT | PROTEIN NITROGEN MG/G DRY WT | TOTAL NITROGEN MG/$10^6$ CELLS | PROTEIN NITROGEN MG/$10^6$ CELLS | T.N./RNA | P.N./RNA |
|---|---|---|---|---|---|---|---|
| N-1 | 6 | 9.09 | 5.25 | 0.207 | 0.119 | 6.18 | 3.57 |
| N-4 | 6 | 11.83 | 6.40 | 0.275 | 0.149 | 6.68 | 3.62 |
| T-1 | 6 | 8.15 | 4.10 | 0.479 | 0.241 | 5.40 | 2.72 |
| T-5 | 6 | 9.70 | 4.74 | 0.647 | 0.316 | 5.51 | 2.69 |

* Cell number the same as table I.

in protein synthesis in the tumor, as the last column shows. The data in table III represent an average of 6 separate batches of tissue. These samples were from the same lots used in table II.

Table IV presents the data obtained with the determinations of the phosphate content of the various fractions resulting from the extraction of the nucleic acids by the technique of Ogur and Rosen (11). Fraction I is the cold alcohol soluble fraction; fraction II is the hot alcohol-ether soluble fraction; and fraction III is the cold acid soluble fraction. The remaining fractions are the RNAP and DNAP. Once again it is evident that on a per-cell basis the tumor has 1.5 to 2.5 times as much phosphate as the normal tissue, and that the amount of phosphate goes up as the tumor growth rate slows. However, there is no clear relationship between the phosphate concentration of the normal tissue and growth rate. The data indicate that, in this instance, the normal tissue remains more constant.

One further observation is important. From all these data it becomes clear that the per-cell basis is quite different than the dry weight basis. Attempts to interpret these data on a dry weight basis would often lead to completely contradictory conclusions.

These data suggest the possibility that the difference between the normal and tumor tissue is due to polyploidy. Therefore, studies were made of the tissues to determine whether there was any difference in chromosome number. These studies were made in 3 ways: measurements of nuclear volume, counts of the nucleolar number, counts of the chromosome number. The irregular shape of the nuclei makes impossible any accurate determination of nuclear volume with the techniques available to us at present. However, it is possible to estimate the volumes by making measurements of the largest diameters and computing the associated volumes on the assumption that we are dealing with a sphere. The figures for the estimated volume were derived in this way. The chromosome counts are similarly difficult as a result of the relatively high chromosome number ($2N = 40$) and the small size of the chromosomes. For this reason, only an average number can be given for these cells. The data for these studies are presented in table V. It can be seen that the data indicate that there is no difference in chromosome number between the normal and tumor tissue under these conditions, and that the tissues have the normal diploid number. However, it should be noted that from these data have been eliminated several cases from the colchicine treated material in which the chromosome number approximated 80. The reason for this was that it was impossible to determine whether these cells represented true tetraploids or merely cases of chromosome separation. The small size of the chromosomes makes such determinations difficult. At best these represented 1 % of the cells. Similarly, the nucleolar number is the same, and very constant throughout the tissues. The slight difference in nuclear volume may be a result of the irregular shapes or may be a reflection of the difference in DNA content. It should be noted at this point that there are undoubtedly a few polyploid cells in the tumor tissue as suggested by chromosome counts, and multiple nucleoli. However, in older tissue and in tissue treated with colchicine, the most common abnormality is not a polyploid cell with a single nucleus, but a multinucleate cell with normal, diploid nuclei (by nuclear size and nucleolar number.) These cells are extremely rare in the types of tissues studied for this paper. They cannot contribute any appreciable amount of DNA to the determinations.

TABLE IV

CHANGES IN PHOSPHATE LEVELS IN 5 FRACTIONS OF NORMAL AND CROWN GALL TISSUE

| FRACTION | No. TESTS | BASIS* | N-1 | N-4 | T-1 | T-5 |
|---|---|---|---|---|---|---|
| I | 4 | mg P/g d.w. | 0.530 | 0.558 | 0.338 | 0.415 |
| I | 4 | mg P/$10^6$ cells | 0.012 | 0.013 | 0.020 | 0.028 |
| II | 4 | mg P/g d.w. | 0.334 | 0.278 | 0.208 | 0.247 |
| II | 4 | mg P/$10^6$ cells | 0.008 | 0.006 | 0.012 | 0.016 |
| III | 4 | mg P/g d.w. | 0.769 | 0.619 | 0.399 | 0.592 |
| III | 4 | mg P/$10^6$ cells | 0.018 | 0.014 | 0.023 | 0.039 |
| RNAP | 4 | mg P/g d.w. | 0.375 | 0.438 | 0.305 | 0.364 |
| RNAP | 4 | mg P/$10^6$ cells | 0.008 | 0.012 | 0.018 | 0.024 |
| DNAP | 4 | mg P/g d.w. | 0.255 | 0.259 | 0.235 | 0.225 |
| DNAP | 4 | mg P/$10^6$ cells | 0.006 | 0.006 | 0.014 | 0.015 |

* Cell number the same as table I.

## DISCUSSION

The data presented in this paper indicate that there is no difference in the response of the tumor and normal tissue to the azaauxin in so far as the nucleic acid, nitrogen, and phosphorus levels are concerned. In both tissues inhibition of the growth with azaauxin resulted in at least slight increases in RNA, DNA, total nitrogen, and protein nitrogen. Only in the case of the acid soluble phosphorus does there seem to be an indication of a difference in response. This differ-

ence is too small to cause any alteration, at this time, of the general conclusion that both types of tissue respond in a qualitatively similar manner.

More important, it is apparent from the data presented on the effect of altered growth rate that there is an intrinsic difference in the 2 types of tissue which cannot be overcome by alterations of the growth rate of the order of magnitude achieved in these studies. It is interesting to note that in all cases the values (per cell) for the tumor tissue run about twice those of the normal, even though in 1 case (N-1 vs. T-5) the growth rate is about the same. These data are in agreement with those of Lee (10), obtained with grape tissue, and Klein (7). Lance (9) reports different relations between normal and tumor tissue of salsify on a dry weight basis. However, table III indicates that dry weight and per cell bases may show wide discrepancies. Therefore, the discrepancies between various studies done on such material may rest solely in the use of a dry weight basis. It seems that the data presented in this paper support the conclusion that there is an intrinsic difference between the tumor and the normal tissue and that these data, taken in conjunction with those of others, indicate that the tumor contains twice as much nitrogen and phosphorus (of various fractions) as the normal.

Perhaps the most striking feature of these data is the difference in the DNA content per cell. The tumor has about 2.5 times as much as the normal. Furthermore, the amount of DNA changes with the azaauxin-induced growth rate, the magnitude of the increase varying from 3 to 40 % depending upon the assay method used and the type of tissue. These findings are not in agreement with the general view that the chromosomal nucleic acid is quite constant from cell to cell. The weight of evidence for this view is overwhelming, and the limited findings with the tumor tissue cannot be taken in contradiction. However, there are other possibilities. It was observed by Pierce (13) that the size of the chromosomes of Viola depended upon the amount of phosphate present, and that plants grown in an excess of phosphate had larger than normal chromosmes. Skoog (18) has demonstrated a large increase in DNA as a result of changes in the auxin levels of cells. Klein (8) has shown that the DNA content of cells increases during the induction phase of crown gall tumors, and Partanen (12) has indicated that older cultures of fern gametophyte tumor tissue show an increase in the DNA content. All of these findings, like those data presented in this paper, indicate that under certain special conditions the DNA content of cells can be altered.

The changes described above may be explained in several ways. One of the most obvious is that a polyploid condition has arisen in the tissue under study. This is the interpretation which Partanen (12) puts upon his data, and it is suggested in a modified form by Skoog (18). However, this is clearly not the case in Pierce's study, nor does it seem to be in the case in the crown gall tumor induction. The increase in DNA observed by Klein using chemical methods was not found by Swift in a cytochemical analysis of the same material (7). Furthermore, Klein (personal communication) has had cytochemical studies made of a strain of Parthenocissus crown gall, which indicate that the cells are very uniform in their DNA content and are diploid. Preliminary analysis of this same tissue in this laboratory indicates that it differs in no way from the tissue assayed and reported in this paper. Finally, data in table V clearly indicate that there is no large amount of polyploidy in the cultures used in this study. This agrees with our work on Klein's tissue. It should be emphasized here that the highest chromosome number ever encountered in tissue was 80 and that such cells were rare. There is no indication that the difference in DNA results from some cells contributing a large amount of DNA. Furthermore, it should be noted here that studies comparing tumor tissue grown in the absence of auxin and normal tissue grown in the presence of *a*-naphthalene acetic acid (usual growth conditions) have shown that the difference in DNA content remains constant. Therefore, 3 possibilities remain: the chromosomal DNA remains constant with a change in some material which behaves like DNA during extraction and assay; the chromosomal DNA varies without any change in the chromosome number; or the extraction procedure removes more DNA from the chromosomes of the tumor than from the normal tissue. The data obtained by others using the same extraction technique (11) indicate that the 3rd possibility is very unlikely. There is evidence for both of the other hypotheses.

TABLE V

CYTOLOGICAL COMPARISON OF NORMAL AND CROWN GALL CULTURES

| TISSUE | NO. CELLS COUNTED | NORMAL | NO. CELLS COUNTED | CROWN GALL |
|---|---|---|---|---|
| Chromosome Number | 163 | 39.9 ± 0.04 | 181 | 39.9 ± 0.06 |
| % cells with no more than 40 chromosomes | | 95.1 | | 80.2 |
| Nucleolar Number | 1000 | 1.05 ± 0.0002 | 1000 | 1.07 ± 0.0008 |
| % cells with no more than 1 nucleolus | | 95.1 | | 93.8 |
| Nuclear Diameter ($\mu$) | 203 | 19.30 ± 0.17 | 200 | 22.47 ± 0.26 |
| Range | | 15.60 to 24.24 | | 16.00 to 30.30 |
| Associated Volumes ($\mu^3$) | | 1170.3 | | 1576.3 |

Skoog (18) has summarized most of the evidence for the 2nd possibility. In his review, he points out that Naylor has direct cytochemical evidence for alteration of the DNA content of the nucleus when meristem cells are exposed to auxin or have auxin withheld. These changes may reach the level of tetraploidy in some cases. Nevertheless, under normal conditions the cells go on to divide without any indication of polyploidy. The work of Pierce (13) is direct support for environmental alteration of chromosomal DNA content. Two important facts emerge from these data: the DNA change can be detected cytochemically (feulgen, etc.) and the withdrawal of auxin from meristem tissue causes an increase in DNA.

The work of Klein (7) on the induction of the crown gall indicates that an increase in DNA can occur without being detected cytochemically. Indeed since, as explained above, at the present time there seems to be no evidence of extra DNA in the tumor of cytochemical detection, the data presented in this paper seem to require such an explanation. These data suggest that the extractable DNA and the chromosomal DNA may represent 2 different quantities. This is further suggested by the observation that the amount of extractable DNA may decrease when bean embryos are grown in the presence of certain concentrations of 2,4-D (unpublished data). The change in DNA content suggests that there is a fraction of DNA present in the nucleus which can be altered by a change in the growth rate. Such material need not be attached to the chromosomes in a highly polymerized state and therefore might not appear as chromosomal DNA in analyses made by feulgen staining of whole nuclei. Further, it might be suggested that under certain conditions (excess phosphate (13), auxin imbalance (18), etc.) this extra-chromosomal material could be incorporated into the chromosome, at least during division; or under other conditions (tumor induction) nucleic acid entering the non-chromosomal pool could exchange with chromosomal DNA to cause transformation. In this respect it is interesting to note that the very condition which might cause increased incorporation of DNA into the chromosomes (increase in growth substances) is necessary for tumor induction.

## Summary

Crown gall and normal tissue were grown in tissue culture in the presence of 7-azaindole propionic acid. On a per-cell basis, the tumor tissue has about twice as much nitrogen and phosphorus as the normal, and about twice as much protein and nucleic acid. As the growth rate is altered, the normal and tumor tissue maintain this difference, but the absolute amount of protein and nucleic acid increases in each. The difference in the DNA content of the 2 tissues and the alteration of the content resulting from a change in the growth rate is not a result of changes in chromosome number. The data presented here suggest the possibility of an increase in the amount of non-chromosomal DNA.

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# PHYSIOLOGY OF GROWTH AND REPRODUCTION IN RICE. II. EFFECT OF PLANT GROWTH SUBSTANCES ON THREE WINTER VARIETIES[1]

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The effects of auxins on whole plants have been studied by many workers. Cereals (1, 2, 6, 8, 25, 26) have been frequently used in such investigations. The recent reviews by Lang (7) and Liverman (11) bring out interesting relationships between the auxin response and the photoperiodic nature of the experimental plant. Auxins frequently promote flowering in long-day plants but seldom in short-day species (8, 27). Rice (*Oryza sativa* L.) is interesting in this connection because the species includes a large number of varieties varying in their normal length of vegetative period (4); autumn varieties have in general a short vegetative period and winter varieties a long one. The winter category may be subdivided into smaller groups with a gradual increase in their vegetative period, namely early-winter, mid-winter, late-winter, and very-late-winter. The autumn, i.e. early and medium-early, varieties are generally long-day or intermediate in their photoperiodic response (13, 14, 16, 17, 19) while the winter ones are predominantly short-day plants (12, 15, 18), though quantitatively there is great variation in photoperiodic response between the various winter groups. With a view to gaining more knowledge of the physiology of the winter varieties, the present investigation has been designed to study the effect of 3 plant growth substances on the growth and reproduction of 3 different winter varieties of rice. A preliminary report on part of this material has appeared elsewhere (21).

## MATERIAL AND METHODS

Seeds of homozygous pure strains T. 1145 (early-winter), T. 141 (mid-winter) and J. 192 (late-winter) were procured from the Central Rice Research Institute, Cuttack. After a preliminary selection for uniformity, the seeds were soaked separately in 3 different concentrations, 500, 250 and 100 ppm (parts per million) of each of the 3 plant growth substances, indole-3-acetic acid (IAA), α-naphthaleneacetic acid (NAA) and 2,3,5-triiodobenzoic acid (TIBA) for 72 hours. The seeds thus treated were washed thoroughly in water and then sown in seed bed pots containing a mixture of soil and cow-dung manure in the proportion of 8 : 1. At the age of 21 days the seedlings were transplanted to 10 inch earthenware pots containing the same mixture of soil and manure. The plants were next subjected to weekly foliar spray with auxin solutions of the same strength as those in which the seeds had been soaked. The spraying was commenced with 4-week-old seedlings and was continued until panicle emergence. There were 6 pots in each treatment, with 4 plants per pot; 30 cc. of the growth substance solution was used to spray 24 plants during each spraying operation. The plants both of the control and treated series were grown from seeding to heading stage under the natural day length (13-hour 14-minutes in July to 10-hour 55-minutes in December) prevalent at Cuttack.

## EXPERIMENTAL RESULTS

TIME FROM SOWING TO EAR EMERGENCE: The average number of days from sowing to ear emergence under different treatments are presented in table I and the analysis of variance in table II. Observations were made each day at about 9 A.M. The data as a whole show that IAA at 250 and 100 ppm, and TIBA at all concentrations, brought about a significantly earlier ear emergence than in the controls. However, there is a marked difference among the varieties in the response to the same growth substance. The auxin IAA had no effect on ear emergence in the early-winter and mid-winter varieties but produced a significant earliness in the late-winter variety at the concentrations of 250 and 500 ppm. The other auxin, NAA, brought about a significant delay in ear emergence in the early-winter variety at higher con-

TABLE I

TIME FROM SOWING TO EAR EMERGENCE IN THE MAIN SHOOT IN DAYS*

| TREATMENTS | | DAYS FROM SOWING TO EAR EMERGENCE | | | |
|---|---|---|---|---|---|
| | | EARLY-WINTER VARIETY | MID-WINTER VARIETY | LATE-WINTER VARIETY | MEAN |
| Controls | | 105.33 | 113.04 | 121.00 | 113.13 |
| IAA | 500 ppm | 105.46 | 113.33 | 120.17 | 112.99 |
| | 250 ppm | 105.29 | 112.63 | 118.96 | 112.29 |
| | 100 ppm | 105.08 | 112.42 | 119.17 | 112.19 |
| NAA | 500 ppm | 107.08 | 112.58 | 120.21 | 113.29 |
| | 250 ppm | 107.25 | 112.50 | 119.25 | 113.00 |
| | 100 ppm | 106.04 | 112.42 | 119.50 | 112.65 |
| TIBA | 500 ppm | 105.42 | 112.46 | 119.64 | 112.51 |
| | 250 ppm | 105.17 | 112.08 | 119.83 | 112.36 |
| | 100 ppm | 105.33 | 112.17 | 120 25 | 112.58 |

S.E. of a treatment mean = 0.161 and L.S.D. at 1 % level = 0.59

S.E. of an individual mean = 0.279 and L.S.D. at 1 % level = 1.03

* Average of 24 plants. Sowing date; 2/7/56.

[1] Received revised manuscript November 26, 1958.

TABLE II

ANALYSIS OF VARIANCE

| SOURCE | D.F. | S.S. | M.S. | F. | 5 % F. | 1 % F. |
|---|---|---|---|---|---|---|
| Between treatment | 9 | 371.06 | 41.23 | 5.52** | 1.94 | 2.53 |
| Between variety | 2 | 94724.41 | 47362.21 | 6340.32** | 3.06 | 4.75 |
| Variety × treatment | 18 | 636.84 | 35.38 | 4.74** | 1.67 | 2.05 |
| Error | 150 | 1120.67 | 7.47 | | | |
| Total | 179 | 96852.98 | | | | |

** Significant at 1 % level.

centrations, was without any effect in the mid-winter variety, but produced significant earliness in the late-winter variety at the 2 lower concentrations of 250 and 100 ppm. The so-called antiauxin TIBA had no effect on the early-winter and mid-winter varieties but produced significant earliness in the late-winter variety at the concentrations of 500 and 250 ppm.

MORPHOLOGICAL CHARACTERS: Observations on the number of tillers, total number of green leaves, number of leaves on the main shoot and the height of the plants as affected by various treatments were recorded every 2 weeks till the time of ear emergence.

Both NAA and IAA decreased the production of tillers of the young seedlings in all the varieties, but the deficiency was made up toward the later stages. TIBA also decreased the production of tillers in the early-winter variety but increased the tillering in the 2 other varieties, mid-winter and late-winter.

IAA was without any effect on the production of leaves in all the varieties, while NAA slightly decreased the number of leaves at the earlier stages in all the varieties, although the differences disappeared at the older stages. TIBA decreased the number of leaves in the early-winter variety, more conspicuously at the younger stages, but increased the number in both the mid-winter and late-winter varieties, particularly in the latter.

IAA and NAA decreased the number of leaves on the main shoot in the early-winter and mid-winter varieties, but had no effect in the late-winter variety. TIBA decreased the number of leaves on the main shoot in the early-winter variety, was without any effect on the mid-winter variety, and increased the number of leaves in the late-winter variety.

IAA brought about a decrease in height in the early-winter variety, was without any effect in the mid-winter variety, and produced an increase in height in the late-winter variety. NAA similarly decreased the height in the early-winter variety, but in the other 2 varieties, although the height was decreased at the younger stages, it was increased considerably over the controls at the mature stage. TIBA had no clear-cut effect on height.

GRAIN YIELD: After the grains had matured the panicles were collected separately for each pot, dried in the sun and weighed. The average yield of grain per plant is presented in table III and its analysis of variance in table IV.

TABLE III

GRAIN YIELD PER PLANT IN GRAMS*

| TREATMENTS | | EARLY-WINTER VARIETY | MID-WINTER VARIETY | LATE-WINTER VARIETY | MEAN |
|---|---|---|---|---|---|
| Controls | | 3.43 | 3.33 | 3.48 | 3.41 |
| IAA | 500 ppm | 3.31 | 2.93 | 3.54 | 3.26 |
| | 250 ppm | 3.42 | 3.11 | 3.39 | 3.30 |
| | 100 ppm | 3.23 | 3.07 | 3.22 | 3.17 |
| NAA | 500 ppm | 2.99 | 3.29 | 3.61 | 3.29 |
| | 250 ppm | 2.70 | 3.61 | 4.01 | 3.44 |
| | 100 ppm | 2.97 | 3.90 | 3.91 | 3.59 |
| TIBA | 500 ppm | 3.06 | 3.83 | 4.25 | 3.71 |
| | 250 ppm | 2.84 | 4.27 | 3.94 | 3.68 |
| | 100 ppm | 2.68 | 4.52 | 4.75 | 3.98 |

S.E. of a treatment mean = 0.133 and L.S.D. at 1 % level = 0.49

S.E. of an individual mean = 0.231 and L.S.D. at 1 % level = 0.85

* Average of 24 plants.

TABLE IV

ANALYSIS OF VARIANCE

| SOURCE | D.F. | S.S. | M.S. | F. | 5 % F. | 1 % F. |
|---|---|---|---|---|---|---|
| Between treatment | 9 | 10.39 | 1.15 | 3.59** | 1.94 | 2.53 |
| Between variety | 2 | 17.19 | 8.60 | 26.88** | 3.06 | 4.75 |
| Variety × treatment | 18 | 21.11 | 1.17 | 3.67** | 1.67 | 2.05 |
| Error | 150 | 48.69 | 0.32 | | | |
| Total | 179 | 97.38 | | | | |

** Significant at 1 % level.

The clearest result is that only TIBA at 100 ppm brought about a significant increase in grain yield, IAA and NAA having no marked effect. TIBA produced a grain yield higher than the controls at 250 and 100 ppm in the mid-winter variety and at 100 ppm in the late-winter variety.

COMPONENTS OF YIELDS: The number of panicles per plant, length of panicle, number of spikelets and of grains per panicle, percentage of grain setting and weight of 1000 grains are presented in table V.

The higher grain yield obtained by treatment with TIBA was mainly due to a significant increase in the number of grains formed per panicle and a higher percentage of seed setting. NAA increased the length of panicle, number of spikelets and of grains formed per panicle in the late-winter variety but was unable to effect an increase in total yield because it reduced very considerably the number of panicles formed per plant.

## Discussion

The responses of the 3 varieties to the 3 growth substances show some clear differences. Thus there was no change in time of flowering in the early-winter variety on treatment with IAA and TIBA, while NAA produced a delay. The mid-winter variety did not respond to any of the growth substances. Only the late-winter variety came to earlier heading as a result of the treatment with all 3 substances. Thus it is clear that TIBA behaves opposite to NAA but akin to IAA in the early-winter variety and in a similar manner to both the auxins in the mid-winter and late-winter varieties. The fact that the same auxin, e.g. NAA, caused delay in flowering in the early-winter variety, no change in flowering in the mid-winter variety and earlier flowering in the late-winter variety points to an interdependence between auxin response and the normal flowering period; the early-winter variety has the shortest vegetative period and the late-winter variety the longest. In other words, auxins only bring about earlier ear emergence when the vegetative period is long. The action of NAA in delaying flowering in the early (20) and early-winter varieties may be due to either a disappearance (7) or an inhibition (23) of the floral stimulus in these varieties, whereas the action of the same auxin in bringing about the opposite effect of earliness in the late-winter variety may be ascribed to a promotion of the formation or accumulation of the flowering stimulus.

Although the 3 winter varieties are grouped photoperiodically in the same category, namely as short-

Table V

Components of Yield*

| Treatments | | Early-winter variety | Mid-winter variety | Late-winter variety | Mean | Early-winter variety | Mid-winter variety | Late-winter variety | Mean |
|---|---|---|---|---|---|---|---|---|---|
| | | *Number of panicles per plant* | | | | *Length of panicle in cm* | | | |
| Controls | | 4.17 | 4.67 | 4.33 | 4.39 | 20.16 | 19.24 | 22.49 | 20.63 |
| IAA | 500 ppm | 4.21 | 4.17 | 4.25 | 4.21 | 19.85 | 19.22 | 22.79 | 20.62 |
| | 250 ppm | 4.29 | 4.50 | 4.00 | 4.26 | 19.71 | 19.08 | 22.88 | 20.55 |
| | 100 ppm | 4.13 | 4.71 | 3.88 | 4.24 | 19.91 | 19.87 | 23.09 | 20.62 |
| NAA | 500 ppm | 3.71 | 3.58 | 3.54 | 3.61 | 19.72 | 20.03 | 23.46 | 21.07 |
| | 250 ppm | 4.08 | 4.42 | 3.54 | 4.01 | 19.37 | 19.12 | 23.80 | 20.76 |
| | 100 ppm | 4.36 | 4.33 | 3.71 | 4.13 | 19.08 | 19.50 | 23.55 | 20.71 |
| TIBA | 500 ppm | 3.92 | 4.75 | 4.64 | 4.44 | 19.81 | 19.56 | 22.47 | 20.61 |
| | 250 ppm | 4.49 | 4.71 | 4.58 | 4.59 | 19.28 | 19.51 | 22.71 | 20.50 |
| | 100 ppm | 4.42 | 4.08 | 4.06 | 4.19 | 19.24 | 19.89 | 23.20 | 20.77 |
| | | S.E. of a treatment mean = 0.100 | | | | S.E. of a treatment mean = 0.141 | | | |
| | | L.S.D. at 1 % level = 0.37 | | | | L.S.D. at 1 % level = 0.52 | | | |
| | | S.E. of an individual mean = 0.173 | | | | S.E. of an individual mean = 0.245 | | | |
| | | L.S.D. at 1 % level = 0.64 | | | | L.S.D. at 1 % level = 0.90 | | | |
| | | *No. of spikelets per panicle* | | | | *Number of grains per panicle* | | | |
| Controls | | 115.64 | 133.80 | 165.56 | 138.33 | 40.91 | 43.09 | 57.63 | 47.21 |
| IAA | 500 ppm | 117.83 | 137.93 | 175.09 | 143.62 | 38.88 | 41.86 | 60.84 | 47.19 |
| | 250 ppm | 110.60 | 131.88 | 180.60 | 141.03 | 40.62 | 39.72 | 61.33 | 47.22 |
| | 100 ppm | 117.13 | 124.27 | 189.81 | 143.74 | 39.51 | 38.21 | 61.77 | 46.50 |
| NAA | 500 ppm | 112.29 | 143.88 | 183.03 | 146.40 | 40.01 | 54.04 | 74.17 | 56.07 |
| | 250 ppm | 106.49 | 122.20 | 195.41 | 141.37 | 33.90 | 45.58 | 80.20 | 53.23 |
| | 100 ppm | 103.87 | 135.19 | 184.86 | 141.30 | 33.71 | 50.51 | 75.55 | 53.26 |
| TIBA | 500 ppm | 113.70 | 130.63 | 166.90 | 137.07 | 39.04 | 46.83 | 67.35 | 51.07 |
| | 250 ppm | 105.61 | 131.26 | 159.94 | 132.27 | 32.36 | 53.51 | 61.30 | 49.05 |
| | 100 ppm | 104.78 | 135.77 | 184.34 | 141.63 | 31.02 | 63.58 | 85.21 | 59.94 |
| | | S.E. of a treatment mean = 2.712 | | | | S.E. of a treatment mean = 1.924 | | | |
| | | L.S.D. at 1 % level = 10.00 | | | | L.S.D. at 1 % level = 7.10 | | | |
| | | S.E. of an individual mean = 4.697 | | | | S.E. of an individual mean = 3.332 | | | |
| | | L.S.D. at 1 % level = 17.33 | | | | L.S.D. at 1 % level = 12.29 | | | |
| | | *Percentage of grain setting* | | | | *Weight of 1000 grains in g* | | | |
| Controls | | 35.53 | 31.94 | 34.67 | 34.04 | 20.48 | 16.88 | 13.65 | 17.00 |
| IAA | 500 ppm | 33.05 | 30.38 | 34.69 | 32.71 | 20.17 | 16.80 | 13.80 | 16.92 |
| | 250 ppm | 36.97 | 29.67 | 34.10 | 33.64 | 19.50 | 17.39 | 13.82 | 16.90 |
| | 100 ppm | 33.95 | 30.73 | 32.40 | 32.36 | 19.77 | 17.07 | 13.63 | 16.82 |
| NAA | 500 ppm | 35.52 | 37.31 | 40.39 | 37.74 | 20.38 | 18.02 | 13.83 | 17.41 |
| | 250 ppm | 31.78 | 37.43 | 41.05 | 36.75 | 19.59 | 17.96 | 14.15 | 17.23 |
| | 100 ppm | 32.43 | 37.47 | 40.93 | 37.00 | 20.11 | 17.77 | 14.01 | 17.30 |
| TIBA | 500 ppm | 34.32 | 35.69 | 40.05 | 36.69 | 20.31 | 17.32 | 13.58 | 17.07 |
| | 250 ppm | 30.63 | 40.87 | 38.37 | 36.62 | 19.51 | 16.95 | 14.08 | 16.85 |
| | 100 ppm | 29.62 | 46.67 | 46.20 | 40 83 | 19 51 | 17.48 | 13.84 | 16.94 |
| | | S.E. of a treatment mean = 1 055 | | | | S.E. of a treatment mean = 0.118 | | | |
| | | L.S.D. at 1% level = 3.89 | | | | L.S.D. at 1 % level = 0.44 | | | |
| | | S.E. of an individual mean = 1.827 | | | | S.E. of an individual mean = 0.204 | | | |
| | | L.S.D. at 1 % level = 6.74 | | | | L.S.D. at 1 % level = 0.75 | | | |

* Average of 24 plants.

day plants, their response to the same short photoperiod, 10-hour or 8-hour, shows great quantitative variation, the early-winter variety showing only a small response, the mid-winter variety a greater response and the late-winter variety the greatest response (5, 12, 18), a behavior which, though not exactly parallel to the effect of the auxins on these varieties, has some similarities with it. The differences between the effects of applied auxin and of short photoperiods are that in the former treatment the earliness was observed only in the long-duration variety, while the degree of earliness was also much less than that caused by short days. The lack of response of the early-winter variety to the auxins so far as flowering is concerned is similar to that of the autumn variety reported earlier (20).

The earlier heading of the late-winter variety as a result of treatment with auxins corroborates qualitatively the results of Sircar and Kundu (24) but differs markedly from their results in that the earliness obtained in the present investigation is very small compared with the earliness of 18 to 27 days observed by them in the late-winter variety, Rupsail of Bengal. As a matter of fact, so great a hastening of flowering induced by auxin has not yet been reported in any cereal, nor in any other plant except pineapple (3, 22). Furthermore, the observations of Sircar and Kundu with a single variety cannot be considered as representative of the effect of auxins on rice in general, as the same auxin in the present investigation produced different results in different categories of rice.

The statement made by Leopold and Thimann (9) in their studies on Wintex barley and teosinte, that both flowering and growth are inhibited by higher concentrations of auxin, is only partially true in the case of the rice plant, since such an inhibition in higher concentrations was observed in the early-winter variety, while just the opposite effect, namely promotion, was noted in the late-winter variety.

The late-winter variety, which is a short-day species, is another addition to the list of short-day plants like cocklebur and Biloxi soybean (27) in which a promotion of flowering has been observed as a result of auxin application. The main difference between rice and the other short-day species is that very high auxin concentrations like 100 to 500 ppm are effective in rice, whereas concentrations as low as 1 to 5 ppm are effective in the other short-day species (27). Thus the flowering of short-day plants in general is not inhibited by auxin, as was suggested by Lang (7).

A general tendency towards poor vegetative growth was observed in all the plants of the early-winter variety treated with the growth substances. The production of tillers and leaves and the elongation of the shoot were conspicuously less in the plants treated with NAA and TIBA solutions, while no marked changes in vegetative characters resulted from treatment with IAA. The results of this investigation are similar to the findings of Leopold and Thimann (10), who found no promotion of vegetative buds by higher concentrations of auxin in Wintex barley and teosinte. In mid-winter rice the suppression of vegetative characters was conspicuous in the seedling stages but gradually disappeared as the plants grew older; indeed the plants treated with NAA showed a significant increase in the height at the mature stage over those of the controls. A similar behavior in plants treated with NAA was observed in the late-winter variety. Thus the plants treated with auxins, especially with NAA, grew faster towards the later stages and this effect was more marked with the late-maturing varieties. Thimann and Lane (26) observed a promotive after-effect on vegetative growth of wheat and oats when treated with IAA which may be similar to this delayed growth acceleration of rice.

The earliness induced by growth substance treatment in all these cases, though statistically significant, is of no agricultural importance.

With regard to the effect of growth substances on reproduction, it is seen that TIBA produced a higher grain yield in the mid-winter and the late-winter varieties, due mostly to an increase in the number of grains formed per panicle and a higher percentage of seed setting. Thus TIBA behaved differently in these 2 varieties than in the early variety (20) and the early-winter variety. But this effect of TIBA on the later varieties is similar to its effect on wheat where an increased grain yield was observed (1). The 2 auxins, more particularly the NAA, brought about an increase in the number of spikelets formed per panicle. Since the spikelets are the later developments of the flower primordia, it may be said that the auxins brought about an increase in the number of flower primordia in rice, a result which is somewhat similar to those of Leopold and Thimann (10) and Hussey and Gregory (6) in Wintex barley.

## Summary

The effect of 3 plant growth substances, IAA, NAA and TIBA, at 500, 250 and 100 ppm upon the growth and reproduction of 3 winter varieties of rice has been studied. The treatments consisted of a preliminary seed soaking for 72 hours followed by a weekly foliar spray, beginning at the 4th week and continuing until ear emergence. The study has led to the following conclusions:

1. IAA had no effect on flowering in the early-winter and mid-winter varieties, but brought about a significant earliness in flowering in the late-winter variety. It somewhat delayed vegetative growth in the initial stages in all the varieties, but the longer duration varieties largely recovered. It had no effect on grain yield or the components of yield.

2. NAA brought about a significant delay in ear emergence in the early-winter variety, had no effect in the mid-winter variety, and produced significant earliness in flowering in the late-winter variety. It delayed vegetative growth at first, but subsequently the height of the treated plants increased over that

of the controls in the mid-winter and late-winter varieties. The number of spikelets and grains per panicle and the grain plumpness were favorably influenced, but there was no increase in total grain yield because the number of panicles formed per plant was decreased.

3. TIBA was without any effect in the early-winter and mid-winter varieties but brought about a significant earliness in the late-winter variety. It increased the production of tillers and leaves in the mid-winter and late-winter varieties at mature stages. It had no effect on grain yield in the early-winter variety, but induced a higher yield at lower concentrations in mid-winter and late-winter varieties by increasing the number of grains formed per panicle and the percentage of seed-setting.

We are grateful to Utkal University for award of the M.S. Gajapati Fellowship to one of us (G. S.) and for a grant-in-aid from the Jnan Vijnan Parishad Fund, to Sri M.P. Jha, Statistician, Central Rice Research Institute, Cuttack, for his advice with regard to the statistical analysis of the experimental data, to Professor B. Samantarai for providing facilities in the Department of Botany, Ravenshaw College, Cuttack, for carrying on this investigation and to Professor Kenneth V. Thimann of the Harvard Biological Laboratories for kindly going through the manuscript and offering many useful suggestions.

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# EFFECTS OF IRON AND CHELATING AGENTS ON DARK CARBOXYLATION REACTIONS IN PLANT HOMOGENATES [1]

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A previous study in this laboratory (12) indicated that leaves and excised roots of bush beans grown under conditions of Fe deficiency fixed larger quantities of $C^{14}O_2$ from an enriched atmosphere than did those receiving adequate amounts of Fe. For this reason, studies have been made to determine the effect of Fe and chelating agents on PEP (phosphoenolpyruvate) carboxylase (1, 9, 13), PEP carboxykinase (9), and the carboxylation enzyme (17) systems that fix $CO_2$.

## MATERIALS AND METHODS

The reagents were prepared for use as follows: The K salt was prepared by dissolving the Ag-Ba salt of PEP in 0.1 M $HNO_3$ and precipitating the Ag and Ba with equivalent amounts of KCl and $K_2SO_4$, respectively, and a stock solution of 0.01 M was prepared in 0.2 M TRIS (tris(hydroxymethyl) aminomethane) buffer, pH 8.0. The K salts of G6P (glucose-6-phosphate) and R5P (ribose-5-phosphate) were prepared from the Ba salt of each by precipitating the Ba with an equivalent amount of $K_2SO_4$. These reagents were centrifuged to remove the precipitates, and stock solutions of 0.02 M were prepared in TRIS buffer as above. Stock solutions of 0.01 M Na salts of ethylenediaminetetraacetic acid (EDTA) and ethylenediamine di(*o*-hydroxyphenolacetic acid) (EDDHA) (6) were prepared and made to pH 8.0.

In the systems studied the addition of 1 micromole ($\mu$M) of EDDHA into the reaction mixtures appeared to provide close to a maximum increase in the rate of the reactions, consequently this level was used throughout the experiments.

Soybean and barley seeds were germinated in sand and allowed to grow about 2 inches in length or about 10 days. The barley seedlings were used directly in the preparation of homogenates. The soybean seedlings were transferred into aerated solution cultures for about 3 weeks with nutrient solutions containing as m.e. per liter, $Ca(NO_3)_2$, 10; $KNO_3$, 1; $MgSO_4$, 8; $K_2SO_4$, 4; and $NH_4H_2PO_4$, 2; and as $\mu$M per liter, $MnSO_4$, 18; $ZnSO_4$, 3.8; $H_3BO_3$, 9.2; and $(NH_4)_6\ MO_7O_{24}$, 0.10. The Fe level was varied to give green plants, moderately deficient plants, and severely deficient plants by the use of $10^{-3}$, $10^{-4}$, and $2 \times 10^{-5}$ M $FeSO_4$, respectively, in the solutions. Trifoliate leaves of equal age were removed from these treatments for assay. Recently matured trifoliate orange leaves which were used in some studies were obtained from trees in the UCLA orchard.

The assays were made as follows: Leaf homogenates were produced by grinding 1 weight of fresh leaves with 1 volume of TRIS buffer, 0.2 M, at pH 8.0, with a mortar and pestle at 0° C. The homogenate was strained through 2 layers of cheesecloth and kept at 0° C until use which never exceeded one half hour. All the reaction mixtures received 140 $\mu$M TRIS buffer pH 8, 20 $\mu$M $KHC^{14}O_3$ containing 90,800 cpm as the $BaCO_3$ precipitate with a conventional thin window Geiger-Müller counter, and 0.1 ml enzyme crude homogenate preparation which was added last. Total volume of all reaction mixtures was 1.0 ml. The mixtures were incubated 10 minutes at 37°C, the enzyme killed with 0.1 ml 1 *N* HCl, which also expelled unreacted $HCO_3^-$, and the mixtures centrifuged. Aliquots of 0.2 ml of the supernatant were then dried in forced air at room temperature in Pyrex or plastic planchets (aluminum and stainless steel planchets were unsatisfactory) and counted with a Geiger-Müller counter. This method was essentially described by Jackson (4) as being a modification of that employed by Bandurski and Greiner (1) and Saltman et al (13).

The coefficient of variability of this method was found to be less than 4 % and this value is taken into account in the discussion of the results. All studies were repeated several times and data are included that represented consistent trends.

Since Fe is known to decarboxylate $\beta$-ketodicarboxylic acids (14), the following procedure was used to insure a measurement of the true effect of Fe on the PEP carboxylase system: Ethanol and DPN were added to the reaction mixtures to furnish sufficient DPNH to convert a maximum amount of the OAA (oxalacetate) formed to malate. After the 10-minute incubation period, 0.1 ml of 2,4-dinitrophenylhydrazine in 1 *N*-HCl was added to each reaction mixture to convert any OAA present to the hydrazone form. Such a system was found to almost completely prevent decarboxylation of labeled products during the period the planchets were dried prior to counting. Protein determinations were made with Folin's reagent (7).

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TABLE I

EFFECT OF Fe PRETREATMENTS ON DARK FIXATION OF $CO_2$ IN HOMOGENATES FROM SOYBEAN LEAVES WITH DIFFERENT SUBSTRATES

| REACTION SYSTEM* | PRETREATMENT | | |
|---|---|---|---|
| | VERY DEFICIENT Fe | MODERATELY DEFICIENT Fe | ADEQUATE Fe |
| | | *cpm* | |
| Blank + Mg | ... | 94 | 44 |
| ATP + Mg | ... | 75 | 40 |
| PEP + Mg | 303 | 600 | 381 |
| PEP+Mg+EDDHA | 567 | 692 | 677 |
| PEP+ATP+Mn | 510 | 496 | 392 |
| PEP+ATP+ Mn+EDDHA | 507 | 489 | 393 |
| G6P+ATP+Mg | ... | 330 | 118 |
| G6P+ATP+ Mg+EDDHA | ... | 543 | 223 |
| R5P+ATP+Mg+TPN | 778 | 490 | 232 |
| R5P+ATP+Mg+ TPN+EDDHA | 1240 | 860 | 1070 |

* The general procedure was outlined under Materials and Methods. The amounts of the other cofactors and of substrates where indicated were as follows: PEP, 1 $\mu$M; G6P, 4 $\mu$M; R5P, 2 $\mu$M; ATP, 4 $\mu$M; TPN, 0.2 $\mu$M; EDDHA, 1 $\mu$M; Mg, 20 $\mu$M; Mn, 6 $\mu$M. It was found previously that the PEP carboxykinase reaction proceeded as well in the crude homogenates with ATP as with ADP. Endogenous ATP+Mn resulted in some, but slight activity only.

## RESULTS AND DISCUSSION

Table I shows the results of experiments in which soybeans were grown at 3 Fe levels. In general all reactions studied resulted in greater activity at a moderately deficient Fe level than at an adequate Fe level. Sometimes with a chelating agent, however, this was not so. At a very deficient Fe level the PEP carboxylase reaction was decreased, while the PEP carboxykinase reaction was essentially unchanged. Under the same condition the reactions with R5P as the substrate were increased; however, a consistent decrease occurred as the Fe level in the treatments increased. Assays of the homogenates for total protein indicated that the results would not be materially different on that basis of calculation. These effects of Fe could be analogous to some of the enzyme activities obtained by Nicholas and Goodman (10) for 4 different Fe levels where, in some systems, they showed increases for moderate deficiency and decreases for extreme deficiency.

Fixation of $CO_2$ with R5P as a substrate is quite probably through the reaction catalyzed by the carboxylation enzyme with ribulose diphosphate as a substrate (17). If this were the case then the phosphoriboisomerase (2) and the phosphoribulokinase (2) enzymes were active in the crude homogenates. The inhibitory effect of Fe actually could have been on any of the 3 enzymes involved.

The fixation of $CO_2$ with G6P as a substrate is suspected to be through the same reaction as R5P. In all aspects studied here and in many unpublished experiments this appeared to be so. Actually, in the 1st experiments made by us with G6P, G6P with TPN and Mn was used as a means of driving the fixation of $CO_2$ catalyzed by isocitric dehydrogenase with $\alpha$-ketoglutaric acid as a substrate (11). It was soon found, however, that more $CO_2$ was fixed with G6P when $\alpha$-ketoglutaric acid was absent than with, and that Mg rather than Mn resulted in greater activity.

TABLE II

EFFECT OF EDDHA, GSH AND TPN ON DARK FIXATION OF $CO_2$ THROUGH R5P IN HOMOGENATES FROM BARLEY LEAVES

| REACTION SYSTEM* | CPM |
|---|---|
| ATP+Mg | 27 |
| R5P+ATP+Mg | 424 |
| R5P+ATP+Mg+TPN | 444 |
| R5P+ATP+Mg+EDDHA | 660 |
| R5P+ATP+Mg+EDDHA+TPN | 805 |
| R5P+ATP+Mg+GSH | 647 |
| R5P+ATP+Mg+GSH+TPN | 807 |

* The procedures were outlined under Materials and Methods and in table I. Amount of GSH was 1.0 $\mu$M.

Since then we have used G6P as a substrate for $CO_2$ fixation with the crude homogenates. Likewise, it was found that $CO_2$ fixation resulting from G6P hindered efforts to demonstrate $CO_2$ fixation through "malic" enzyme with pyruvate as a substrate (11). In addition to the Mg requirement the responses of the G6P and R5P reactions were parallel for ATP, TPN, and GSH (glutathione) as cofactors and were parallel for NaF inhibition. NaF did not inhibit the PEP reactions, but it did inhibit the G6P and R5P reactions. Since the activity obtained when ATP and Mg were added without a substrate was also inhibited by NaF, it is possible that this endogenous reaction, at least in part, was similar to G6P and R5P.

Effects of chelating agents on $CO_2$ fixation are given in tables I, II, III, and IV. In all cases except possibly for the reaction catalyzed by PEP carboxy-

TABLE III

EFFECT OF CHELATING AGENTS ON DARK FIXATION OF $CO_2$ WITH HOMOGENATES FROM TRIFOLIATE ORANGE LEAVES

| REACTION SYSTEM* | CPM |
|---|---|
| Blank+Mg | 23 |
| PEP+Mg | 2180 |
| PEP+Mg+EDDHA | 3630 |
| G6P+ATP+Mg | 536 |
| G6P+ATP+Mg+EDDHA | 647 |
| G6P+ATP+Mg+EDTA | 628 |
| R5P+ATP+Mg | 12900 |
| R5P+ATP+Mg+EDDHA | 14700 |

* The procedures were outlined under Materials and Methods and in table I. Amount of EDTA was 0.1 $\mu$M.

TABLE IV

EFFECT OF Fe AND CHELATE SEPARATELY AND TOGETHER ON DARK FIXATION OF $CO_2$ IN TRIFOLIATE ORANGE LEAF HOMOGENATES

| ADDITIONS IN $\mu$M | REACTION SYSTEM* PEP + Mg | PEP + ATP + Mn | R5P + ATP + Mg |
|---|---|---|---|
| | | *cpm* | |
| Nothing | 1350 | 1580 | 4270 |
| 1 Fe | 1090 | 1320 | 3620 |
| 1 EDDHA | 2010 | 2060 | 4950 |
| 1 Fe + 1 EDDHA | 1320 | 1490 | 4240 |

* The procedures were outlined under Materials and Methods and in table I.

kinase a chelating agent was necessary for maximum activity. This is analogous to the findings of Weissbach et al for the carboxylation enzyme (17). In all cases there was a greater percentage of response to chelate at an adequate Fe level than at a slightly deficient Fe level. This provides some evidence that the effect of the chelating agent is in chelation of Fe. Additional evidence for this is in table IV, where the addition of EDDHA to the reaction mixture overcame the inhibiting effect of the Fe added, while added Fe overcame the enhancing effect of the chelate. Chelating agents increased activity also for homogenates from barley and soybeans. These data also suggest a possible reason why synthetic chelating agents have a growth-promoting effect on plants other than by supplying micronutrients as has been reported (8).

It was of interest to note that stimulation with a chelating agent on the PEP carboxylase reaction as observed in several experiments was less consistent with roots than with leaves.

Part of the chelating agent effect with R5P in table I may be the result of TPN which was included in the reaction mixture with and without the chelating agent. In a large number of tests it was shown that when TPN was added to a reaction mixture containing R5P+Mg+ATP it had no effect, but did have a consistent enhancing effect when added to R5P+Mg+ATP+EDDHA (table II).

To further test the inhibitory effect of Fe, it was added to reaction mixtures with PEP, G6P, and R5P as substrates. Each was found to be significantly inhibited (table V).

To determine the nature of the inhibitory effect of Fe, it was added to reaction mixtures containing PEP+Mg, and double reciprocal curves were plotted. The enzyme was a crude preparation from trifoliate orange leaves. The curves in figure 1 indicated that Fe may be a competitive inhibitor of PEP carboxylase. This observation is at slight variance with that of Walker (16), who found that $10^{-3}$ M Fe completely inhibited the PEP reaction in certain Crassulacean plants by 100% precipitation of the enzyme. In the present work Fe at $4 \times 10^{-2}$ M did completely inactivate the system from trifoliate orange leaf.

TABLE V

EFFECT OF Fe ON DARK FIXATION OF $CO_2$ IN TRIFOLIATE ORANGE LEAF HOMOGENATES

| REACTION SYSTEM* | CPM |
|---|---|
| Blank + Mg | 34 |
| PEP + Mg | 2360 |
| PEP + Mg + Fe | 1050 |
| ATP + Mg | 135 |
| ATP + Mg + Fe | 66 |
| G6P + ATP + Mg | 536 |
| G6P + ATP + Mg + Fe | 164 |
| R5P + ATP + Mg | 12300 |
| R5P + ATP + Mg + Fe | 6820 |

* The procedures were outlined under Materials and Methods and in table I. Six micromoles of Fe as $FeSO_4$ were added where indicated.

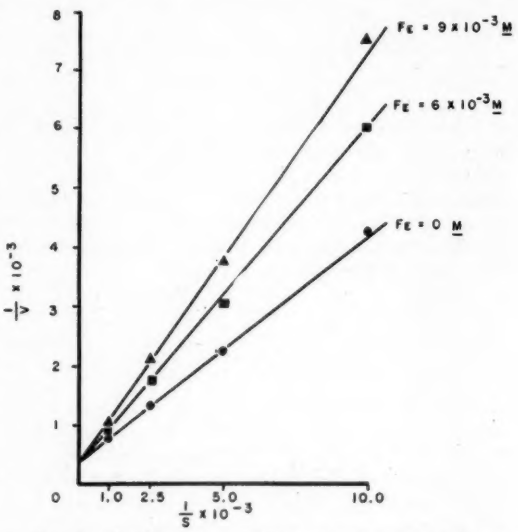


FIG. 1. Double-reciprocal plot of the effect of Fe on $C^{14}O_2$ fixation by crude homogenates of trifoliate orange leaves through the reaction catalyzed by PEP carboxylase. S is molar concentration of PEP in the reaction mixture, and V is cpm $C^{14}$ in 0.2 ml reaction mixture. Fe as added as $FeSO_4$.

The indication that PEP carboxylase activity is greater in plants in which Fe is insufficient may offer an explanation of the observations that certain organic acids (3, 12), K, and sometimes Ca (15) are increased in Fe-deficient plants. As previously stated, dark fixation of $CO_2$ by this system leads directly to organic acid formation and accumulation in some plant species (1, 9, 13, 16). This in turn may induce increased cation uptake (4, 5). The observation that extreme Fe deficiency resulted in less $CO_2$ fixa-

tion than did a moderate Fe deficiency which in turn resulted in more $CO_2$ fixation than did an adequate Fe level may explain the observations that in lime-induced chlorosis, organic acid contents increased and then decreased as the disorder advanced (3, 12).

## Summary

Fixation of $CO_2$ with PEP (carboxylase and carboxykinase systems each included) and R5P as substrates was greater in homogenates of plants grown at a slightly deficient Fe level than in those grown at an adequate Fe level. For severe Fe deficiency the fixation catalyzed by PEP carboxylase was decreased. A chelating agent sometimes increased the amount of $CO_2$ fixation with the PEP carboxykinase reaction. A chelating agent was necessary in the reaction mixture for maximum activity of the reaction catalyzed by PEP carboxylase as well as for the carboxylation enzyme. Addition of Fe to assay mixtures inhibited $CO_2$ fixation through PEP. Kinetic studies indicated this to be a competitive inhibition. Fe also inhibited $CO_2$ fixation when R5P and G6P were used as substrates and activity was increased with both substrates by use of chelating agents.

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# LOSS OF PHOSPHORUS-32 BY PLANT ROOTS AFTER FOLIAR APPLICATION[1,2]

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The fact that plant roots are capable of losing ions to the ambient medium was recognized as early as the nineteenth century, and has been the subject of intermittent attention since that time. Excellent reviews of the early work in this field were compiled by Merrill (10) and True (14). Definite conclusions in these early experiments were often lacking since the work was limited solely to the use of electrolytic or chemical techniques. Such techniques prevented accurate determination of very low concentrations, as well as detection of ion movement in a direction opposite to that of net flow. Recent development of radioisotope methods has made such measurements possible, and has provided a new approach to the study of ion loss by roots.

Evidence exists to indicate that root loss of ions may play an important role in the overall nutrient

[1] Received revised manuscript November 28, 1958.

[2] This paper is based on work performed under contract no. AT(30-1)-2117, Project 281, with the U.S. Atomic Energy Commission.

economy of the plant. Such loss was of sufficient magnitude to be detected chemically in the plant growth medium (2, 5, 7), and to cause a significant reduction in the nutrient content of the plant top (1). Regulated loss of ions by roots has been envisoned as one means by which the plant is able to maintain electrical neutrality while differentially absorbing companion ions (7, 8). Considerable use has already been made of root loss determinations in the development of "free" and "outer" space concepts, and the volumetric measurement of such space (3). In these and related studies loss of phosphorus, sulfur, selenium, rubidium, and other elements by roots to the external medium was noted. The forces and mechanism involved in such transfers are not clearly understood, however, and invite continued investigation.

The present work was undertaken to study the effect of phosphorus in the root environment and of time on loss of radiophosphorus from roots. Test plants were placed in a graded series of phosphorus solutions and also in water, and the effect of such treatments on root loss of $P^{32}$ was studied over long (6 to 7 day) and short (50 minute) time periods. Experimental techniques differed in 3 important respects from classic excised root methods often employed in studies of this type, and these warrant brief initial mention. Firstly, whole intact plants were used so that any possible effect of plant top on the phenomenon under study would not be lost. Secondly, the radioactive material was introduced to the plant through the foliage in order to prevent contamination of the root surface with isotope. Lastly, complete nutrient solutions instead of single salt solutions were employed in order to minimize root loss of materials other than those under observation.

## Materials and Methods

Bean seeds of the variety Wade were germinated on damp cheesecloth until the radicles were about 2 to 3 cm long, and then moved for further growth to a device designed to eliminate root injury in later transfers. Seeds were placed on a paper towel which was held taut on a wood frame and which was suspended approximately 75 mm above the surface of a reservoir of aerated water. A hole was provided in the towel for each radicle so that it could grow downward into the water. Plants were removed from this system when they were about 8 cm in height by saturating the towel with water and peeling it away from the plants. In this and subsequent manipulations, extreme care was taken not to injure the roots.

The young plants were next moved to a controlled environment facility and grown in 2 liters of aerated complete nutrient solution for 4 days. Solutions were then discarded, roots rinsed with water, and new treatment solutions added. Radioactive phosphorus was applied to the surface of 1 of the primary leaves of each plant immediately following the solution change. The area of application was approximately 4 $cm^2$, and was previously outlined with a black water emulsion paint not toxic to plants. Extent of root loss of foliar applied isotope was estimated 6 to 7 days after application. Ten ml of nutrient solution from each plant container was evaporated to dryness in a stainless steel planchet and assayed for radioactivity. Just prior to the time the solution samples were taken, the test plants were removed from the solution and also prepared for counting. The foliar area upon which the isotope was originally placed was removed as a leaf plug, and the remaining plant top tissue was placed in a crucible, dried, and weighed. The tissue was finally ashed at 450° C until gray, and then assayed for radioactivity. Roots were treated separately in the same manner as the plant tops except that dry weights were not taken.

The controlled environment facility consisted of an enclosure 16 by 20 feet in which light intensity, light duration, day temperature, and humidity were controlled. A day length of 14 hours with a light intensity of 1200 ft-c 1 foot above the plant containers was maintained in all studies. Day temperatures fluctuated 8 degrees around a mean of 29° C. and dropped to between 10 and 15° C at night. Relative humidity was kept at 50 %.

The radioactive phosphorus was obtained from the Oak Ridge National Laboratory as $H_3P^*O_4$ in dilute hydrochloric acid. Acid normality of the various shipments ranged between 0.42 and 0.58. Specific activity of all shipments was above $1 \times 10^5$ millicuries per gram phosphorus. Dilutions were made of the original shipments so that 25 microcuries of carrier free isotope were applied to each plant in 0.3 ml solution. Adjustments in pH of the acid isotope solution were not made since greatest foliar uptake of phosphorus occurs at low pH values (13). Treated leaves were held horizontal with a wire arm to prevent running off of isotope.

Two systems of counting were necessitated by the vast differences in activity between the solution and tissue samples. Plant tissues were counted in the crucible in which they were ashed, using a thin end-window Geiger-Müller tube (1.4 mg/$cm^2$). Where activity was beyond the capacity of the instrument, a 5.4 mg/$cm^2$ filter was placed between the sample and end-window. Results obtained with the filter are so identified in the text. All solution samples were counted in a gas-flow counter equipped with an ultra-thin end-window (150 $\mu$g/$cm^2$). Both Geiger Müller and gas-flow counters were connected to a conventional amplifier-scaler-high voltage system. It is important to emphasize here that it was not possible to calibrate the different instruments used against a common source so that results for the solution and tissue samples are not comparable.

Sixteen phosphorus treatments were studied ranging from 0 to 14 milliatoms phosphorus per liter solution. Treatments are designated in the text according to the milliatoms phosphorus in 1 liter of solution. The fact was mentioned earlier that treatments were not single salt solutions but contained a balanced compliment of essential mineral elements. Content of all elements except phosphorus was held constant

among treatments (N = 18 milliatoms/l; K = 6; Ca = 6; Mg = 1; S = 1). Microelements were added according to Hoagland (6). Changes in $H_2PO_4^-$ were accommodated by alterations in the $NH_4^+$ to $NO_3^-$ ratio. The pH value for fresh solutions ranged between 6.5 and 6.8.

Tap water also served as a treatment. Chemical tests for calcium, potassium, and phosphorus in the tap water proved negative. Flame methods were used for the bases, while the colorimetric ammonium molybdate method was used for phosphorus. Electrical resistance of the tap water was $2.2 \times 10^4$ ohms, as compared with $7.9 \times 10^4$ for demineralized water. The pH of tap water was 6.5 which was similar to that of demineralized water.

A record of solution pH changes was maintained during the treatment periods early in the investigation, but was eventually abandoned because changes were not considered to be of sufficient magnitude to be nutritionally important. In addition, non-metabolic root loss of ions of the type studied here has been shown to be virtually insensitive to pH (3).

The overall set of 14 treatments was divided into 4 groups, each of which was studied at a different time. Each treatment was replicated 8 times, and plants were arranged in randomized block order. Results were analyzed according to conventional analysis of variance methods.

## Results

Long Duration Experiment: Radioactivity of treatment solutions at the conclusion of the experiment is shown in figure 1. Results indicate that root loss of $P^{32}$ did occur to the graded series of solutions and that this loss increased with increasing stable phosphorus in the root environment. Loss in this series appeared as an exponential function of environmental phosphorus. Radiophosphorus was also lost to tap water at a level slightly greater than that to the 0.0 P treatment.

The phosphorus economy of a plant is known to have an influence on foliar uptake and distribution of this element in the plant (13). A check was therefore made to determine whether nutrient treatments affected foliar uptake of $P^{32}$ in the present experiment, and whether this was a factor to consider in interpreting the root loss data. Tops and roots of the test plants were assembled according to treatment at the conclusion of the experiment, and assayed for $P^{32}$ according to procedures outlined. Results for the run in which water was a treatment are given in table I.

Table I

Differences in Radiophosphorus Content of Roots and Tops of Bean Plants

| | Treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| | $H_2O$ | 0.0 P | 0.2 P | 0.4 P | 0.6 P | 0.8 P | 1.0 P |
| | *Radioactivity as counts per minute* * | | | | | | |
| Roots | 284** | 2863 | 2542 | 2608 | 2136 | 2884 | 2204 |
| Plant tops | 1095** | 3461 | 2754 | 3036 | 3084 | 2910 | 3785 |

* Counts made through a metal filter.

** Statistically significant at the 5 % level. LSD 0.05 roots—838; LSD 0.05 tops—1083.

No statistically significant differences in activity occurred between the graded phosphorus series of treatments, either for root or for top samples. Loss data for these plants plotted according to tissue activity yielded essentially the same curve as that in figure 1. Tissue activity for the water treated plants was considerably below that of the phosphorus group, however, and indicated a relatively lower foliar uptake of isotope by these plants. A replotting of loss data according to plant top activity elevated the water treated plants to a level of that of the 0.8 P plants, while a recalculation based on root activity elevated the water plants above all other treatments.

Although solution treatments did not substantially affect foliar uptake of $P^{32}$ (except in the water treatment), it is reasonable to expect that they did influence the overall phosphorus economy of the plants and thus caused a shift in the ratio of stable to radioactive atoms (specific activity) in the tissue. The shift would be toward a dilution of radioions (a lowering of specific activity) as phosphorus in the treatment solutions increased, and would in effect tend to minimize the loss values for the high phosphorus treatments. Loss of a greater total number of phosphorus atoms would be required for each ordinate unit in figure 1 for the high phosphorus treatments than for the low phosphorus treatments. Treatment differences in root loss could therefore be expected to be somewhat greater than those given in figure 1, were absolute values available.

Dry weights of the plant tops were taken to determine whether root loss results were conditioned by growth differences among plants. In no case were weight differences among treatments statistically significant.

Short Duration Experiment: *Methods and results*: In the interest of simplicity of presentation, solution activity was regarded up to now as a direct indicator of the extent to which roots lost $P^{32}$. The existence of such a simple relationship may be questioned when critical examination is made of the test conditions of the experiment. Six to seven days elapsed from the time of isotope application to the time root loss determinations were made. During this time, solutions in which the test plants were grown were not changed. Opportunity was therefor ample for recapture by plants of a portion of the $P^{32}$ atoms once lost to the solution. Ion recapture of this type would tend to be greatest for the low phosphorus and least for the high phosphorus treatments. A true picture of ion loss by roots is further complicated in the earlier experiments by possible influences of solution treatment on the specific activity of the plant tissue.

A second study was undertaken under conditions designed to largely eliminate these experimental weaknesses. Root loss was measured over much shorter

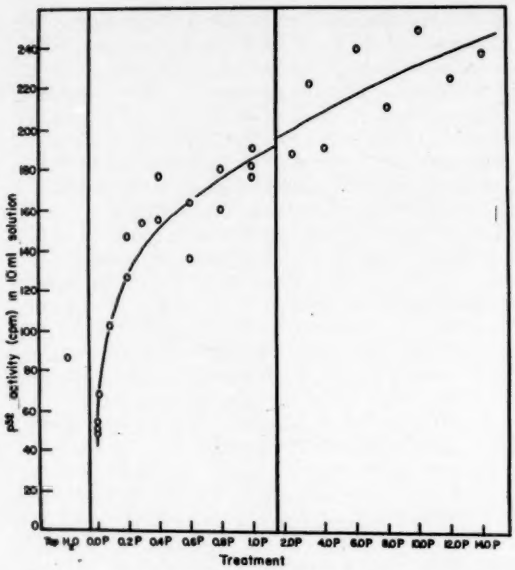


FIG. 1. Loss of foliar applied $P^{32}$ from roots of bean plants to tap water as well as to a graded series of phosphorus solutions. Each point is the average of 8 plants. Treatments are designated according to milliatoms stable phosphorus/liter solution.

time periods than those employed earlier, thus avoiding prolonged exposure of the plants to the different phosphorous regimes. In addition, treatment solutions were periodically drained and renewed throughout the test period to reduce the chances of recapture by the root of ions which had previously passed out of the root.

Experimental procedures for starting plants were similar to those reviewed. Bean plants were germinated and then grown in 2 liters of aerated complete nutrient solution. The method and activity of isotope application was also similar. The complete nutrient solutions were not discarded upon $P^{32}$ application, however, but plants were allowed to remain in them for 2 more days. Plants were then individually removed from the solutions, and the roots lowered into glass tubes 10.0 cm long and 2.5 cm in diameter. Tubes were sealed at the lower end except for a solution drain and an air inlet. At the time of plant transfer, each tube contained 30 ml of the old complete nutrient solution.

The old solution was next drained from each tube and replaced with 30 ml of treatment solution. Solutions were again drained and renewed at 5, 15, 30, and 50 minute intervals, using the first drainage as the base time. All tubes were vigorously aerated throughout the course of the study. Four ml of each drainage was evaporated to dryness in a stainless steel planchet and counted to determine extent of root loss of $P^{32}$ to the solution.

Treatments were 0.0 P, 0.4 P, 1.0 P, 6.0 P, and were again designated according to milliatoms stable phosphorus in a liter of solution. Tap water and demineralized water also served as treatments. Electrolyte content and pH values for the 2 waters were given earlier.

The experimental procedures were carried out twice, each treatment being replicated 4 times in the 1st run and 6 times in the 2nd run. Results for the 2 runs were identical in virtually every respect, and were therefore composited for presentation in figure 2.

Results indicate that $P^{32}$ was lost by the test plants throughout the entire course of the study. Greatest loss occurred in the 1st 5 minutes of the study, and decreased exponentially with time. Solution treatments did not affect the exponential nature of the curve, but did influence the level at which it occurred. De-

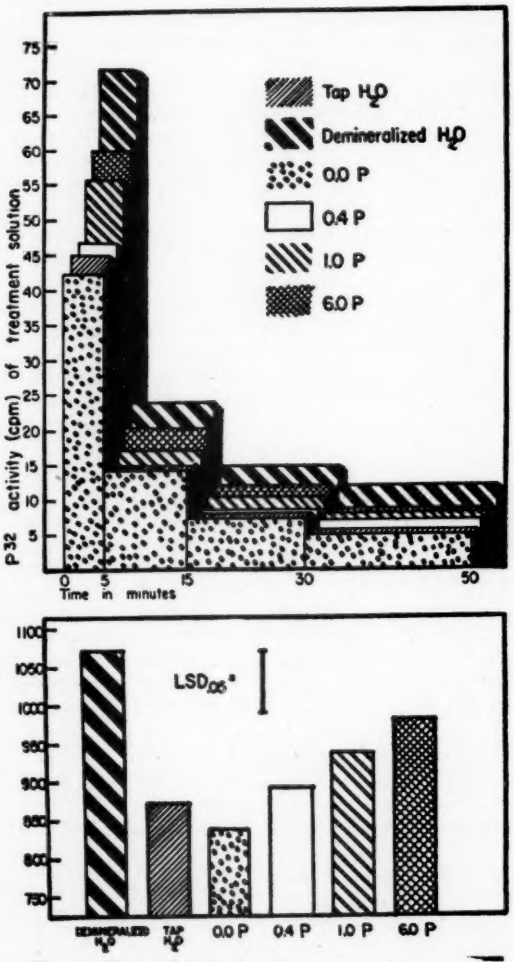


FIG. 2. Loss of radiophosphorus from bean roots to various solutions over a 50 minute period. (*Top*) Loss per minute throughout 4 intervals of the total period. (*Bottom*) Total loss to each treatment for the entire test.

mineralized water showed the greatest loss, followed in order by the 6.0 P, 1.0 P, 0.4 P, tap water, and 0.0 P treatments. Treatment differences remained consistent throughout the entire study. Results for the phosphorus series of treatments showed loss of $P^{32}$ as a function of stable phosphorus in the root environment and are in agreement with those of the long term experiment given in figure 1.

## Discussion

The results indicated clearly that radiophosphorus passed from the root to the external medium. These findings are not in themselves new, and have been reported by others (1, 5, 11, 12). The present experiment departed from techniques generally employed in such studies, however, in that introduction of isotope was made through the foliage and not the root of the plant. Surface contamination of roots with radioactive material during the introduction process, and subsequent release of the contaminant during loss studies, was thus avoided. All radiophosphorus which entered into the root loss calculations came originally from the root interior, having at one time or another followed the sequence of foliar absorption and internal distribution. Exact definition of terms as tissue "interior", "exterior", and root "surface" in this instance is difficult in light of conflicting opinions regarding root morphology as it applies to transport of ions. Definitions may vary according to the school of interpretation favored. If, as in the concept of "free" space, the interface barrier to free diffusion of ions in the root is taken as a natural line of division, root "exterior" could be designated on the one hand as any area external to the physical structure of the root itself or again, as certain areas extending to the endodermis (8), or even to within the stele of the root (4).

Root loss of phosphorus was conditioned in this experiment by composition of the root environment. Referring for the present only to the graded phosphorus series of treatments and not to water, loss occurred as a positive function of ambient phosphorus concentration. A similar relationship was shown by Hevesy (5) for wheat plants and suggests that an isotopic exchange reaction is largely involved in the loss process. Such a system of isotopic exchange would indicate that root phosphorus occurs to a considerable extent in the bound rather than the freely diffusable state. These conclusions are in agreement with Overstreet et al (12) who showed a non-metabolic binding of newly absorbed phosphorus in barley roots. The exponential nature of the loss in the present experiment further suggests that differences exist in tenacity with which individual phosphorus ions are maintained in the bound state.

Loss of radiophosphorus to the water treatments cannot, however, be reconciled with the proposed model of isotopic exchange. Loss was often greater to water which was virtually free of exchange materials than to nutrient solutions which were abundantly supplied with such materials. The fact that loss to demineralized water was significantly different than that to tap water adds further to the difficulty of arriving at a reasonable explanation for the water treatments.

The present results and also those by Overstreet et al (12) showed an effect of time on the magnitude of phosphorus loss. Such a time effect is difficult to interpret since it could occur as a product of various and unrelated phenomena. A diminishing root content of total phosphorus during the test period, or at least that fraction which is easily exchangeable, could result in a drop in root loss values with time. A time effect could further be explained as a function of distance the exchange components necessarily traversed into and out of the root tissue to complete the loss process. A gradual recession into the root interior of the reaction responsible for the large scale release of $P^{32}$ as equilibrium between stable and radioions was approached in the exterior regions, could effectively reduce root loss of radioions with time.

The above interpretations should be considered in light of 2 important limitations in experimental procedures worthy of emphasis. Firstly, recapture of $P^{32}$ atoms by roots after they had once passed out of that tissue, was not prevented entirely, even in the short term experiment. Ion recapture was possible during the time intervals between solution drainings, although the frequency with which solutions were changed undoubtedly reduced the incidence of recapture. Recapture of radiophosphorus was also possible between the time of isotope application and actual utilization of the plant in root loss studies. A buildup of $P^{32}$ on the root surface could have occurred in this manner and influenced subsequent loss figures, especially in the early time periods of the study. Influences of ion recapture such as these could not in any respect be compensated for in the present experiment, and root loss figures must therefore be considered apparent and not absolute throughout.

Secondly, while root loss of $P^{32}$ was considered here exclusively as a root phenomenon, such loss may indeed involve forces residing in tissues outside of the root. A differential replenishing of root phosphorus by aerial tissues during the test period could well have occurred, for instance, to influence the root loss results. That aerial tissues do indeed influence loss results has already been demonstrated by Jacobson et al (8).

## Summary

Root loss of $P^{32}$ to a graded series of phosphorus solutions and to water was studied over a long (7 day) and a short (50 minute) time interval. Intact bean plants were used in which the isotope was introduced through the foliar tissue.

In the long term experiment, loss to the phosphorus solutions occurred as an exponential function of ambient phosphorus concentration. Foliar uptake of radiophosphorus was conditioned by the water treatment so that loss values for this treatment varied according to the mode of calculation used.

Loss to the graded phosphorus solutions also occurred as an exponent of ambient phosphorus in the short term experiment. Loss to demineralized water was considerably greater than that to tap water. Loss to all treatments was greatest in the 1st 5 minute period of the study, and decreased exponentially with time. Solution treatments did not affect the basic nature of the time-loss curve, but did influence the level at which it occurred.

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# GERMINATION OF LIGHT SENSITIVE SEED IN CROSSED GRADIENTS OF TEMPERATURE AND LIGHT

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Seed germination is strongly temperature dependent and is also frequently influenced by light. These variables are interrelated: the relation of germination to temperature is dependent on the light treatment and conversely the relation of germination to light treatment is dependent on the temperature. (2, 4, 8, 9) Using standard methods, a complete study of the interrelation of these 2 variables, on the rate or attainable percentage of germination of a single strain of seed, requires the correlation of a large number of separate measurements. It seemed likely that the crossed gradients principle might be useful for seed germination experiments.

In this paper we shall describe an attempt to survey, in a single experiment, the effects of combinations of a wide range of temperature and light treatments. This was done with an apparatus developed originally to study algal growth in crossed gradients of temperature and light intensity (6, 7). In the previous work the algae were grown on an 11 × 12 inch agar surface in a shallow chamber with a temperature gradient from left to right and with a light intensity gradient from front to back. Thus a single experiment gave a 2-dimensional picture of the growth response to the 2 variables.

[1] Received December 5, 1958.

Drs. E. H. Toole and S. B. Hendricks suggested that lettuce seed (*Lactuca sativa* L. var. Grand Rapids) would be suitable for testing the utility of the crossed gradients principle for seed germination investigations.

The effect of the interaction of temperature and light on germination in this variety of lettuce has been studied in great detail by several workers. This particular seed is very light sensitive, giving a greater germination percentage in light than in darkness. The photo-response varies with the temperature (3) and the type of radiation—red light promotes and far-red radiation inhibits the germination of water-imbibed seeds (5). The action of the red and far-red radiation is repeatedly reversible (1, 8). Inhibition by far-red radiation is particularly temperature sensitive if seeds are first given a saturating dosage of red light and then exposed to a less than saturating far-red dosage (9). We therefore decided to germinate seeds in a temperature gradient crossed with a far-red radiation

gradient using seed which had been promoted to its maximum germination percentage by exposure to red light.

## Experimental Method

Very little modification of the original apparatus was needed. A light-tight box with a sloping lid to allow condensed moisture to run to one edge was made to fit over the growth chamber in place of the lucite cover used for algal culture. A U-shaped metal strip was screwed to the edge of the box and fitted over the rim of the growth chamber to give a light-tight seal. A sheet metal cover was made to protect the seeds at the top of the growth chamber for a dark control strip. The edge of this cover was bent down and pressed into the cloth to keep out stray light. This cover was also used to protect a 2nd strip exposed to red light but not to far-red radiation.

Red light was supplied by two 15-watt warm white fluorescent tubes 1.5 feet from the growth chamber covered with 2 layers of red cellophane. The transmission of the cellophane rises sharply from zero at 5800 Å to 70 % at 6500 Å. The intensity without the cellophane was 150 ft-c. The fluorescent tubes gave very little far-red. For the far-red radiation the original graded lighting system was used (6, 7) with the tungsten light filtered through 2 layers of red and 2 layers of blue cellophane. The wave lengths of the radiation transmitted were similar to that used by Borthwick et al (2). Manipulation was done in the dark using a flashlight covered with green cellophane which did not affect the seeds.

The light arrangements and exposure times were tested in a preliminary experiment in Petri dishes at 21° C on black cloth over moist filter paper. The following germination percentages were found: Dark, 13.5 %; red light as used in the plate experiment for 15 minutes, 98.5 %; red followed by 1 minute far-red of maximum intensity used on the plate, 3 %.

In Petri dishes the seeds germinated just as readily on 1.5 % agar as on moistened filter paper. However, an agar slab proved to be unsatisfactory for germination in the crossed-gradient apparatus because the agar dried too rapidly at the hot edge of the chamber. The agar was therefore replaced by 2 layers of moistened filter paper. This kept uniformly moist throughout the experiment because the condensed water from the cold edge was drawn across to the hot edge of the paper by capillary action. In the later experiments the filter paper was covered with a square of black cotton cloth. This helped greatly in photographing the final germination pattern as the white roots showed up clearly against the black background (fig 1).

Since the lettuce seeds become light sensitive within 4 to 8 minutes of imbibition (4), they were spread over the dry filter paper (or cloth) and spaced as evenly as possible with a fine brush. Then distilled water was added to the edges of the chamber and also sprayed over the seeds. The light-tight cover was put in place and the seeds were left to imbibe at 21° C in the dark. After 10 hours a strip, 1.5 inches deep, at the back edge of the chamber was covered and the rest of the seeds were exposed to the red light of uniform intensity for 15 minutes to give the maximum promotion of germination (2). The Petri dish experiments mentioned above confirmed that the seeds were fully promoted by the light. A further 1-inch wide strip was covered and the remaining seeds exposed to the far-red radiation gradient for 1 minute. The light-tight cover was then replaced and a temperature gradient ranging from 8 to 30° C was set up.

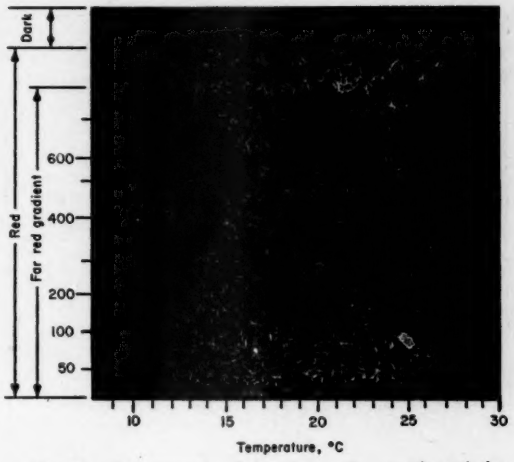


Fig. 1. Photograph of lettuce seeds germinated for 3 days at various temperatures. The top strip from left to right was kept dark. The strip below it was given red light for 15 minutes. The rest of the plate was also given the red light exposure and then an exposure of 1 minute to a graded intensity of far-red. The units of far-red intensity are in ft-c with the color filters removed.

The seeds were left to germinate for 2 more days in the dark. A photograph of the seeds was then taken and a distribution diagram of the germinated seeds was made by placing clear cellophane over the chamber and making a mark where each seed had germinated. By making diagrams of each germination experiment and combining these a composite picture which summarized the results was obtained (fig 2).

## Results

The results are based on 3 comparable experimental runs. The number and spacing of the seeds were not exactly the same in each experiment but the general pattern of the response was similar. The general trends of the germination pattern can be seen in a photograph of the growth chamber (fig 1), but there are not sufficient seeds at each position to show up all differences in a single experiment. This is particularly noticeable in the dark control strip where differences become clear only in the summary of several experiments. (Compare figs 1 and 2). The pattern of the germination response to the range of conditions agrees very well with published work on this variety of lettuce. In the dark control, germina-

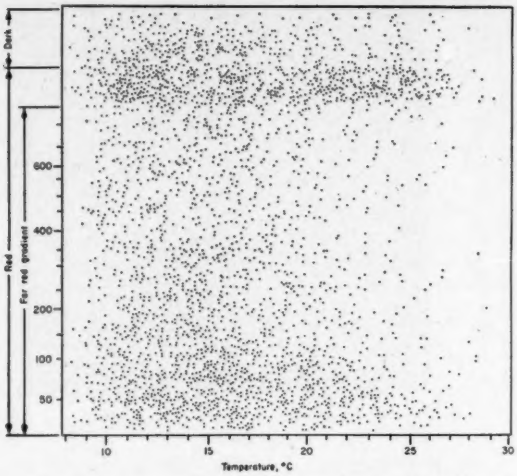


FIG. 2. A summary of 3 experiments like that of figure 1. Each dot represents a germinated seed.

tion increases with decrease in temperature from 28 to 10° C but falls off below 10° C (9). In the strip exposed to the red light, germination is promoted fairly uniformly over the temperature range with a possible fall in germination around 10° C and a sharp fall at about 27° C (9). Previous work (9) has shown that, given a maximum dosage of far-red radiation, the germination percentage drops to near the dark germination percentage, and with less than saturation dosage the amount of inhibition is directly proportional to the dosage. A similar response is shown here where germination is inhibited with increase in temperature and with radiation dosage. Below 100 ft-c the exposure was not sufficient to inhibit germination, except at the higher temperature (fig 2).

Superimposed on the germination pattern is a well marked pattern of root growth which is temperature but not light dependent. Maximum root growth is between 25 to 30° C (fig 1). The different rates of root growth tend to obscure the germination pattern in the photograph of the growth chamber.

## DISCUSSION

The results show clearly that the crossed-gradient principle could be of value in seed physiology studies. The main advantage of this technique over others is that a wide range of conditions can be covered with 1 relatively simple experiment. The crossed gradient apparatus gives the germination response in a 2-dimensional picture which could be obtained otherwise only by a large number of individual tests. The results are qualitative rather than quantitative, but quantitative results could be obtained by counting seeds in selected areas. Perhaps the most useful application of this approach would be to survey the range of temperatures and light sensitivity of untested seeds before more quantitative methods are used.

Very little modification of the apparatus would be needed to study other factors besides those used in this work. Daily alternation of temperature could be produced by periodically reversing the hot and cold water inlets of the apparatus. This would produce a constant temperature in the middle with alternating temperatures on each side and an equal average temperature at all points. The growth chamber could be divided into parallel strips and each strip sown with different seeds. In this way, if the temperature or the light intensity were made uniform over the growth chamber, seeds of different varieties, seeds of the same variety of different ages or from different storage conditions, etc., could be grown in either a light intensity gradient or a temperature gradient.

## SUMMARY

An apparatus designed for growing microscopic algae in crossed gradients of temperatures and light intensity has been used to study the interaction of light and temperature on the germination of light-sensitive lettuce seeds.

The results obtained by this apparatus agree qualitatively with the previous experiments performed by standard techniques.

We wish to thank Drs. E. H. Toole and V. K. Toole for the seeds and for very helpful advice. We also are indebted to Mr. R. W. Hart for keeping the crossed-gradient apparatus in running order.

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# STUDIES ON THE MECHANISM OF STEM GROWTH INHIBITION BY VISIBLE RADIATION [1, 2]

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Applied gibberellic acid specifically reverses the light inhibition of etiolated stem growth in certain species (4, 5). It therefore appears reasonable to suggest that light regulates stem elongation in these plants through some effect (or effects) on the gibberellin metabolism of the plant. Results presented in the present paper provide further support for this hypothesis. The effect of light on the gibberellin metabolism might be mediated through 1 of 3 general mechanisms. First, light may decrease the synthesis of endogenous gibberellin; or, second, it may cause the destruction (or diversion) of the natural gibberellin. Finally, light might make the tissue less responsive to a given amount of gibberellin. The results presented in the present paper make possible a partial resolution of these alternatives.

## METHODS AND MATERIALS

Morse's Progress # 9 (Ferry-Morse Seed Co., Los Angeles), a strongly dwarfed variety of *Pisum sativum* was used for these experiments. The seeds were germinated and grown in vermiculite in plastic cups and continuously subirrigated with deionized water. The plants were grown in a darkroom maintained at 25 ± 1° C and a green safelight was used for watering and experimental manipulations. All plants were treated with gibberellin solutions (as a 4 $\mu$l alcoholic drop) while in the dark. Immediately after treatment those plants which were to be irradiated were transferred to continuous red light (150 ergs·cm$^{-2}$·sec$^{-1}$). Other radiation treatments are described in the particular experiment. A band of red radiation was obtained by filtering the radiation from pink 40-watt G.E. fluorescent tubes through 0.01-inch red cellulose acetate. Far-red radiation was obtained by filtering the radiation from 300-watt incandescent bulbs through Corning red-purple ultrafilters and 0.01-inch red cellulose acetate. The energy was 450 ergs·cm$^{-2}$·sec$^{-1}$ standardized at 730 m$\mu$. The total height of the plants from the soil level to the stem apex was measured in all cases. All experiments reported were repeated 2 or more times with substantially the same results.

## EXPERIMENTAL RESULTS

To determine the time-course of growth following a single irradiation, 6-day-old dwarf pea seedlings were exposed to red radiation (2,000 ergs·cm$^{-2}$·sec$^{-1}$) for various lengths of time and the height of the plants was measured daily. The results of a typical experiment are illustrated in figure 1. It may be seen that even 10-minutes of irradiation results in a marked inhibition of growth. It is noteworthy that the growth inhibition by light continues for a period of several days. After 4 to 5 days the growth rate of the irradiated plants returns to the dark-grown rate, but the growth rate never exceeds that of the dark-grown plants. The same time-relation appears

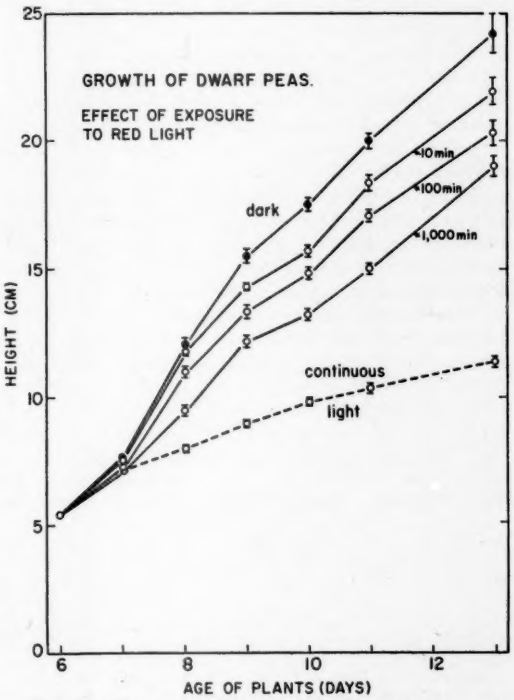



FIG. 1. Time-course of growth of dwarf pea seedlings following a single or continuous high intensity (2,000 ergs · cm$^{-2}$ · sec$^{-1}$) red irradiation. The duration of the irradiation is indicated on the growth curves.

[1] Received December 11, 1958.

[2] Report of work supported in part by the Herman Frasch Foundation.

to hold for the longer irradiations. Continuous irradiation results in a linear rate of growth much slower than the dark controls.

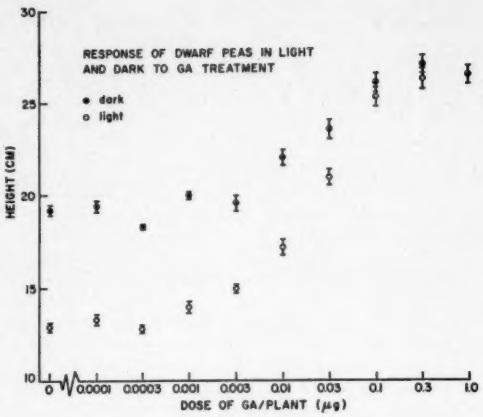
Far-red radiation will completely reverse the effects of brief red irradiation of the dwarf pea (table I). Plants were exposed to 15 minutes of red radiation (2,000 ergs·cm$^{-2}$·sec$^{-1}$) followed, in the cases indicated, by far-red radiation for 30 minutes. The time interval between the 2 irradiations was approximately 10 minutes. It is clear that these plants respond to red and far-red radiation, in terms of total growth, in a manner identical to the response of individual internodes of other species (2, 3).

In order to establish more critically the nature of the interaction between light and gibberellin, quantitative experiments were run comparing the response of the plants to added gibberellin in light and darkness.

Gibberellic acid treatment will prevent inhibition of pea stem growth by radiation (in both dwarf and tall varieties) and it will also reverse the dwarf character in peas as well as other species (1, 4, 8). These facts make it possible to compare directly the gibberellin sensitivity of irradiated dwarf plants with those grown in darkness. The results of a typical experiment are illustrated in figure 2 a. The inhibition as a result of irradiation is obvious at low doses or no gibberellin treatment. At saturating doses of gibberellin the plants attain the same maximum growth whether irradiated or grown in darkness.

In order to compare directly the response of irradiated plants with those maintained in darkness, growth promotion has been calculated as the percent of growth at a saturating dose of gibberellin. The grouped results of 4 separate experiments are illustrated in figure 2 b. In 2 of these experiments gibberellic acid (Eli Lilly and Co.) was used and in the other 2 gibberellin A (Merck and Co., Inc.) was used. No significant difference between the response to gibberellic acid and to gibberellin A was ever observed.

At saturating doses of gibberellin the irradiated and non-irradiated plants are found to be of equal height, while without gibberellin irradiation markedly



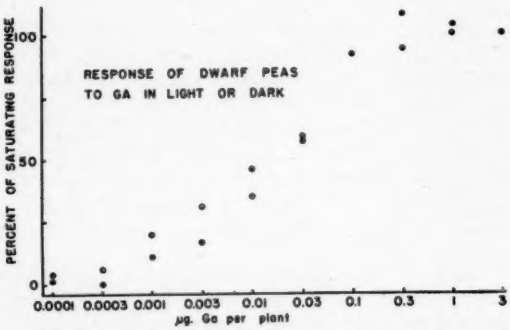




FIG. 2a (*top*). The height of red irradiated and dark-grown dwarf pea seedlings 4 days after treatment with gibberellic acid at the doses indicated.

FIG. 2b (*bottom*). The effect of gibberellin on growth in light (open circles) and darkness (closed circles). The abscissa represents the promotion of growth in each case as a percentage of the response to a saturating gibberellin treatment. Average of 4 separate experiments.

TABLE I

TIME-COURSE OF GROWTH OF DWARF PEA SEEDLINGS FOLLOWING A SINGLE IRRADIATION AS INDICATED BY THE TREATMENT *

| TREATMENT | DAYS AFTER TREATMENT 0 | 1 | 2 | 3 | 4 | TOTAL GROWTH |
|---|---|---|---|---|---|---|
| | *height of plants (cm)* | | | | | |
| Dark | 4.0 ± 0.3 | 6.5 ± 0.3 | 10.6 ± 0.4 | 14.2 ± 0.3 | 17.4 ± 0.3 | 13.4 |
| Red (15 min)** | 4.1 ± 0.2 | 6.5 ± 0.2 | 9.9 ± 0.4 | 12.8 ± 0.4 | 15.6 ± 0.4 | 11.5 |
| Red + far-red (30 min) | 4.1 ± 0.3 | 6.0 ± 0.2 | 10.8 ± 0.3 | 14.1 ± 0.2 | 17.4 ± 0.3 | 13.3 |
| Far-red (30 min)† | 4.0 ± 0.2 | 6.4 ± 0.3 | 10.8 ± 0.4 | 14.3 ± 0.3 | 17.4 ± 0.2 | 13.4 |
| Red (24 hr)‡ | 4.1 ± 0.2 | 5.7 ± 0.3 | 7.5 ± 0.2 | 10.2 ± 0.2 | 12.3 ± 0.4 | •8.2 |

* Also indicated is the standard deviation of the mean. The dark-grown seedlings were treated at the age of 6 days.

** Intensity 2,000 ergs · cm$^{-2}$ · sec$^{-1}$.

† Intensity 450 ergs · cm$^{-2}$ · sec$^{-1}$.

‡ Intensity 150 ergs · cm$^{-2}$ · sec$^{-1}$.

inhibits growth. At intermediate doses of gibberellin, the height of the irradiated plants is less than in darkness (see fig 2 a), but the promotions of growth are equal fractions of maximum growth promotion.

## Discussion

The results illustrated here indicate that the effective natural gibberellin is reduced as a result of irradiation. Successive increments of added gibberellin, then, give response curves in the 2 conditions (dark vs. irradiated) which approach a common maximum value.

If radiation was, in fact, acting on some growth process other than the gibberellin system, or if the radiation acted to reduce the number of active sites for gibberellin action, the growth at saturating doses of gibberellin could not be equal. Furthermore, if radiation acted to interfere with gibberellin action (e.g., causing a competition for active sites or reducing the affinity for active sites) then saturation would not occur at the same gibberellin dose, as is shown to occur. Irradiation results in an approximately 70 % reduction in growth rate under the conditions used here. If the radiation were acting, e.g., by reducing the affinity for gibberellin, then a 50 % reduction in growth rate might result from a 50 % reduction in affinity for the gibberellin, and 2 times as much gibberellin would have to be applied to reach maximum growth. The data are probably not sufficiently accurate to detect such a difference with certainty. However, it should be noted that the observed deviations from the theoretical results are, especially at low doses, in the opposite direction from that expected for an effect on affinity.

Evidence supporting the general view that it is the concentration of gibberellin which is affected by the radiation, rather than its action at the site of growth comes from the work of Idle (3). He has found that irradiation of the tip (in *Vicia faba*) is even more effective in the inhibition of growth than irradiation of the growing region. Furthermore, the effectiveness of irradiation of the tip persists far longer. Gibberellin has been shown to be produced in the stem tip of the pea seedling (6).

These results provide further evidence that irradiation reduces stem growth through a reduction in the level of the natural gibberellin of the plant. This is true for *Pisum sativum* and presumably for other species but in other cases, e.g., Phaseolus (6), the situation is clearly more complex.

In a paper published in 1941, Went reported the results of experiments in which dwarf peas were irradiated briefly with orange light (9). The time-course of growth was not followed, but the average length of each internode as well as total height was determined at the end of the experiment. The results showed that little or no inhibition of stem growth occurred as a result of a single brief irradiation, but the radiation did cause a substantial change in the relative lengths of the various internodes. Generally, that internode which was elongating at the time of irradiation was shorter and the internodes laid down subsequently were longer than in the dark control.

Went's results are subject to 2 different interpretations which could not be resolved at that time. It might be that the light causes a temporary reduction in growth which is later compensated by an increase in the growth rate. This would be reflected in the length of the various internodes. These same experimental results would be observed if the irradiation temporarily stimulated node formation but had no effect on stem elongation. The results presented in the present paper make it clear that this 2nd interpretation is the correct one. The response of these plants is, then, analogous to that of *Phaseolus vulgaris*, in this respect (e.g., (2, 3, 6)).

The present author has suggested in an earlier paper (4) that the endogenous gibberellin might be reversibly inactivated by light. This idea was proposed as the result of the 1st interpretation of Went's results in which it was assumed that brief irradiations resulted in a temporary decrease in growth rate followed by an increase to restore the original height. There is no longer a question of a reversible inactivation of the natural gibberellin. It appears clear that the natural gibberellin is either destroyed (or diverted) or its synthesis is inhibited in response to visible radiation.

It may be suggested that the effect of radiation is probably on gibberellin synthesis. This is indicated by the duration of the response of the plant to brief irradiations. The results reported here show that the effects of a single brief irradiation last as long as 4 or 5 days. Since the steady-state concentration of natural gibberellin in the plant is almost certainly very low (consider the extraction experiments which have been reported, and the fact that these experimental plants are gibberellin-limited dwarfs) it would appear highly unlikely that the long term effects observed could be due to a photo-destruction of the gibberellin present at any one time. Destruction of the gibberellin as a result of irradiation could only be envisaged if the radiation resulted in the establishment of a gibberellin-destroying system which would continue long after the radiation had been removed. This latter possibility cannot, of course, be ruled out.

It may be concluded that visible radiation affects stem growth in such plants as Pisum by reducing the natural gibberellin level, either by causing the formation of a gibberellin-destroying system or by retarding the synthetic processes which result in the formation of gibberellin.

It has been shown here that the red : far-red pigment system is effective in the inhibition of stem growth in this plant. The low intensities utilized for continuous irradiation presumably preclude participation by the "high intensity" pigment system of Mohr (7). Whether growth inhibition as a result of continuous low intensity irradiations is due exclusively to the red : far-red pigment system is not established. However, it is known that gibberellic acid

may prevent stem growth inhibition as a result of both low and high intensity continuous irradiation in this species (1, 4).

## Summary

Exposure of a dwarf pea (Morse's Progress # 9) to a single brief irradiation with red light results in an inhibition of growth which persists for several days. After 4 or 5 days the plants resume growth at the dark-grown rate. Far-red irradiation will negate the red effect.

The effect of a series of gibberellin doses on the growth responses of irradiated or dark-grown peas has been determined. In the absence of applied gibberellin the growth of irradiated plants is inhibited compared to that in darkness. At saturating doses of gibberellin the irradiated and dark-grown plants are the same height. The proportionate responses of irradiated and dark-grown plants are equal at equal doses of gibberellin.

It is concluded that visible radiation probably inhibits stem growth in this species through an effect on the level of endogenous gibberellin.

The author wishes to thank Eli Lilly and Co. for samples of gibberellic acid, and Merck and Co. Inc. for samples of both gibberellic acid and gibberellin A.

The technical assistance of Miss Virginia Gottschall contributed substantially to this work.

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# GROWTH RESPONSES OF ALASKA PEA SEEDLINGS TO VISIBLE RADIATION AND GIBBERELLIC ACID[1, 2]

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Inhibition of stem growth is a general response of etiolated plants to irradiation, but the mechanism of radiation action is still little understood. We know the action spectra for both the low energy (2, 12) and a high energy (8) inhibition of stem elongation. The effective pigments are, however, not yet identified. It has also been shown that the application of gibberellic acid will specifically reverse both low intensity and high intensity radiation inhibition in certain species (4, 6, 11). It was felt that a more detailed description of stem inhibition by visible radiation and its reversal by gibberellic acid in a single species might provide a background for a more complete and general understanding of these phenomena.

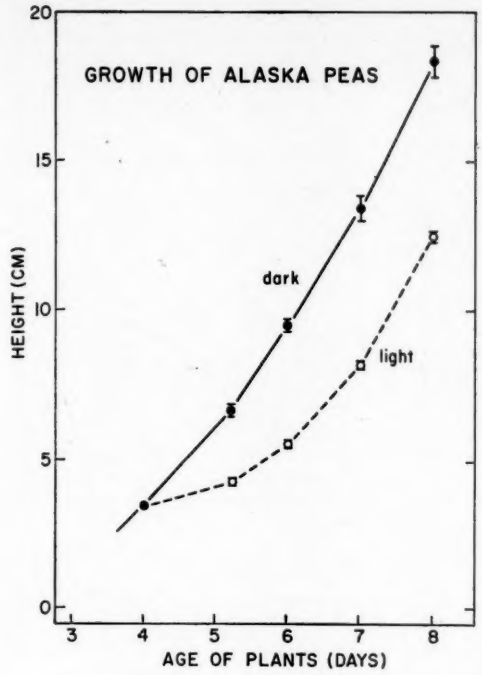
## Materials and Methods

Seeds of *Pisum sativum*, variety Alaska (Ferry-Morse Seed Co., Los Angeles) were soaked in deionized water for 4 to 5 hours in darkness, then planted in vermiculite in plastic cups. The seedlings were grown in a dark room maintained at 25 ± 0.5 C. The pots were continuously subirrigated with deionized water. For the experiments reported here 5 uniform plants were selected per pot and at least 2 pots of plants were used for each experimental treatment. The experimental treatments were usually started when the seedlings were 4 days old, 3 to 4 cm above the vermiculite level.

The sources of radiation were the same as those previously described, i.e., colored fluorescent tubes filtered through colored cellulose acetate. Measurement of radiation intensity was with a calibrated photo-cell, also previously described (6). In the experiments reported here the red and blue radiation were never used simultaneously in the same room. The temperature under the lights was maintained at 25 ± 0.5° C, measured with a mercury thermometer.

[1] Received December 11, 1958.

[2] This investigation was supported in part by the Herman Frasch Foundation.



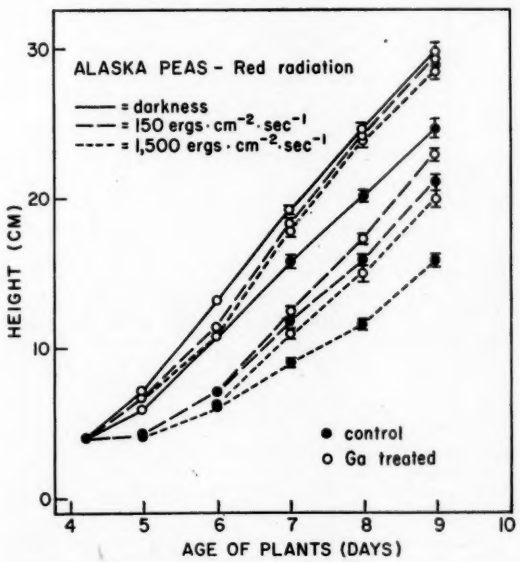




FIG. 1 (*top*). Stem growth of Alaska pea seedlings in continuous darkness and on transfer to continuous low intensity (150 ergs · $cm^{-2}$ · $sec^{-1}$) red light.

FIG. 2 (*bottom*). Stem growth of Alaska pea seedlings in response to continuous low intensity (150 ergs · $cm^{-2}$ · $sec^{-1}$) and high intensity (1500 ergs · $cm^{-2}$ · $sec^{-1}$) red radiation. The effect of gibberellic acid applied at day-0 (upper curves) or on day-2 are shown.

Gibberellic acid (Merck and Lilly) was applied as a 4 $\mu$l ethanolic drop to the growing point at a concentration calculated to give a dose of 1.0 $\mu$g per plant.

## EXPERIMENTAL RESULTS

LOW INTENSITY RADIATION INHIBITION: Transfer of Alaska pea seedlings from complete darkness to low intensity red radiation (e.g., 150 ergs · $cm^{-2}$ · $sec^{-1}$) results in a substantial inhibition of stem growth. Nevertheless, when these plants are irradiated for relatively long times the growth habit appears to be that of a pole-type, climbing plant. Investigation of the time-course of radiation-induced growth inhibition makes possible an explanation of this observation. It may be seen (fig. 1) that on irradiation a marked inhibition occurs, which persists for 24 to 36 hours. Following this period of inhibition the plants resume a growth rate fully equal to that in darkness. Complete recovery requires 48 to 72 hours. Thus, the effect of continuous low intensity radiation is only transient, and the plants soon counteract the effect of the irradiation, so far as stem elongation is concerned. If plants are returned to darkness after 24 hours irradiation, stem growth is almost identical to those which are irradiated for the duration of the experiment.

When gibberellic acid is applied before or at the time the plants are irradiated, inhibition is prevented (fig 2). When gibberellic acid is applied at a later time it prevents further inhibition and immediately restores the growth rate to that of dark-grown plants. It never increases the growth to a rate greater than that of the dark-grown plants. The plants treated after inhibition by radiant energy, then, never attain a height equal to that of the dark-grown plants, but the growth rate is the same.

HIGH INTENSITY RADIATION INHIBITION: When dark-grown Alaska peas are transferred to high intensity red radiation (1,500 ergs · $cm^{-2}$ · $sec^{-1}$) a further effect of irradiation becomes evident. The initial inhibition is identical to that observed with radiation of low intensity. Furthermore, the plants receiving high intensity irradiation show the same recovery from inhibition. However, in this case the growth rate after recovery remains less than that of the dark or low intensity irradiated plants. The specific effect of high intensity radiation, then, results in a slower growth rate following recovery from the initial inhibition.

When gibberellic acid is applied to the plants at the time of transfer to high intensity radiation, the inhibition is completely prevented. This includes both the initial inhibition and the specific high intensity effect on subsequent growth (fig 2). When the gibberellic acid is applied after the initial light inhibition, i.e., 48 hours after irradiation begins, the effect is to promote growth to a rate approaching that of the dark- and low intensity irradiated plants. This is in distinction to the plants in low intensity

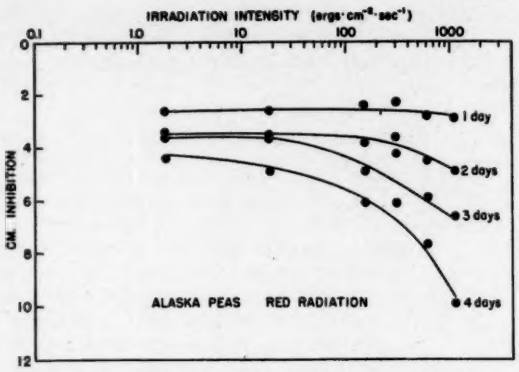



FIG. 3. Inhibition of stem growth by continuous red radiation of various intensities after 1 to 4 days of irradiation.

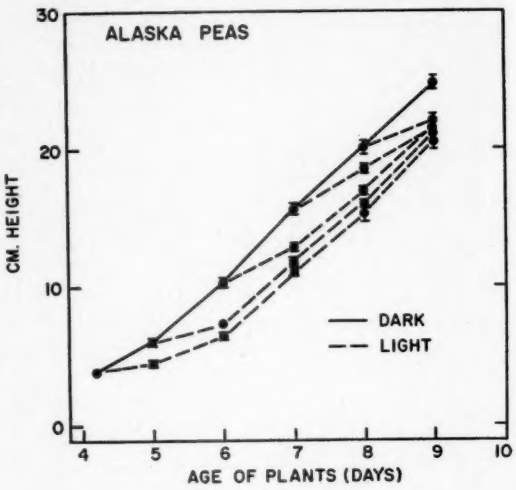
radiation where treatment with gibberellic acid after the initial light inhibition has no effect on the growth rate. However, it is consistent with all the observations reported here, in that gibberellic acid prevents all types of light inhibition in this species.

The effect of exposure of plants to continuous red radiation of various intensities is illustrated in figure 3. The varying intensities were obtained by filtering the colored radiation through neutral (white polyvinyl plastic and brown wrapping paper) filters. As indicated above, after 1 day of irradiation the inhibition was nearly equal regardless of intensity. At the lower intensities little or no further inhibition is observed, while at higher intensities the inhibition continues. The threshold intensity for the high intensity inhibition appears to be approximately 100 ergs · $cm^{-2}$ · $sec^{-1}$. No threshold intensity for the low intensity effect can be estimated from this data. It appears to be of the order of 0.1 ergs · $cm^{-2}$ · $sec^{-1}$ or less.

EFFECT OF SEEDLING AGE ON THE RESPONSE TO IRRADIATION: It has been previously observed that the length of the 2nd internode of Alaska peas was particularly affected by irradiation (12). Perhaps the plants are particularly sensitive at this age, or perhaps this was simply the internode which was expanding at the time irradiation was begun.

Experiments were therefore undertaken in which plants were moved to continuous irradiation at daily intervals and the growth rate was followed thereafter. The results are presented in figure 4. It may be seen that between 4 and 8 days of age the seedlings have substantially the same sensitivity to irradiation. They show the typical recovery phenomenon regardless of age, or the particular node growing at the time of transfer to light. (The 2nd internode expands between the 4th and 5th day of growth.)

EFFECT OF ALTERNATING LIGHT AND DARK PERIODS: The Alaska pea shows a typical inhibition and recovery when grown in continuous, low intensity red radiation. The effect of interrupting irradiation by daily dark periods has also been examined. Daily irradiations of 0, 8, 16 and 24 hours of low intensity red radiation were given and the growth rates were followed. The results are presented in figure 5. It



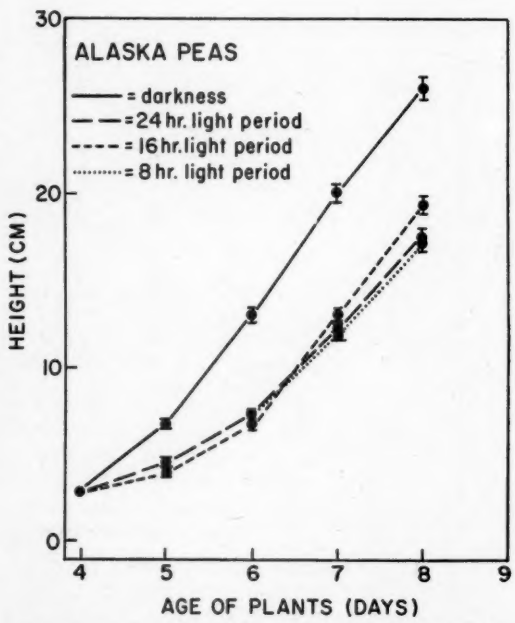




FIG. 4 (*top*). The effect of age of the plant on inhibition by continuous low intensity red radiation.

FIG. 5 (*bottom*). The effect of the length of the daily light period (red radiation, ca. 300 ergs · $cm^{-2}$ · $sec^{-1}$) on light inhibition and gibberellic acid reversal of the inhibition.

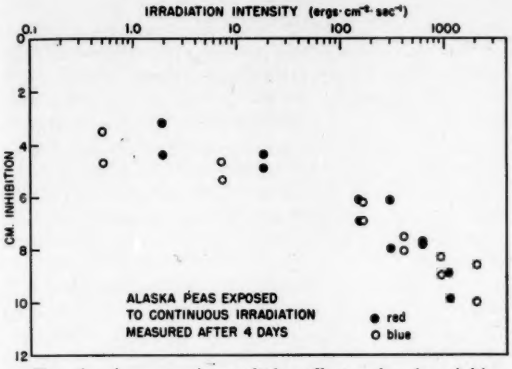



FIG. 6. A comparison of the effects of red and blue radiation of various intensities on the inhibition of stem growth. Included in the figure are the results of 2 separate experiments with red and 2 experiments with blue radiation. The seedlings were 4 days old at the start of irradiation and the measurements were made 4 days later.

is clear that as little as 8 hours of irradiation per day causes the same growth inhibition as continuous irradiation. These results, together with those on the effects of brief daily irradiation demonstrate that the recovery from inhibition is not specific for continuous irradiation but takes place equally readily with daily cycles of light and darkness. When the daily irradiation is further reduced a minimum duration of irradiation is reached (between 8 hours and 10 minutes, see below) when the radiation ceases to be effective.

EFFECT OF BLUE AND FAR-RED RADIATION: In order to understand further the nature of the radiation inhibition several experiments were undertaken observing the daily growth rates of the plants exposed to blue radiation of varying intensities.

The results are presented in figure 6. The effect of blue radiation of 6 different intensities is compared with red at generally comparable intensities after 4 days irradiation. Two experiments with blue and 2 with red radiation are illustrated. Blue radiation appears to have an effect completely comparable to red at the same energy levels.

Since the effects of blue radiation were as great as red, suggesting the high energy response of Mohr (8), the effect of far-red radiation was also investigated. The results of a typical experiment are presented in table I. The plants were transferred to continuous far-red radiation at the age of 4 days and the measurements were made 4 days later. It is clear that far-red radiation, at the energy levels presented (450 ergs · $cm^{-2}$ · $sec^{-1}$) also inhibits stem growth. This inhibition also is negated by gibberellic acid treatment. The inhibition by far-red radiation cannot be due to an effect of high temperature, because the radiation inhibition observed here is prevented by gibberellic acid treatment, while inhibition of Alaska stem growth by high temperature is not prevented by gibberellic acid treatment (7).

LOW ENERGY RADIATION RESPONSE: Several experiments have been performed in order to determine whether the Alaska pea would show a response to brief irradiations with red light. Plants were given either a single irradiation of varying duration or daily irradiations, and the subsequent growth rates were followed.

The growth of seedlings which had been given a single exposure to red radiation is illustrated in table II. It is apparent that there is little influence by as much as 100 minutes of red radiation (2,000 ergs · $cm^{-2}$ · $sec^{-1}$). Daily irradiations of 10 minutes duration had no measurable effect on the growth of these plants. It appears, then, that the low energy red : far-red pigment system either does not regulate stem elongation in this vairety, or the sensitivity to radiation is very low.

RESPONSE OF DARK-GROWN ALASKA PEA SEEDLINGS TO GIBBERELLIC ACID: An observation, reported earlier (4) has been repeatedly confirmed and is indicated in many of the experiments reported here. This is the response of Alaska pea seedlings when grown in complete darkness to applied gibberellic acid. The author can only emphasize that when conditions are such that the seedlings grow from

TABLE I

TIME-COURSE OF GROWTH OF ALASKA PEA SEEDLINGS IN RESPONSE TO A SINGLE TREATMENT WITH RED RADIATION

| TREATMENT | DAYS FROM TREATMENT | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| | *average height of plants (cm)* | | | | | | |
| Dark control | 4.0 ± 0.09 | 6.2 ± 0.11 | 10.4 ± 0.13 | 15.9 ± 0.20 | 21.6 ± 0.25 | 25.6 ± 0.67 | 29.3 ± 0.68 |
| 10 min red | 3.7 ± 0.10 | 5.4 ± 0.12 | 9.4 ± 0.20 | 15.1 ± 0.33 | 20.8 ± 0.44 | 24.9 ± 0.70 | 30.1 ± 0.76 |
| 100 min red | 4.0 ± 0.09 | 5.6 ± 0.13 | 9.8 ± 0.27 | 15.4 ± 0.43 | 21.8 ± 0.49 | 25.9 ± 0.32 | 28.8 ± 0.69 |
| Continuous light | 4.0 ± 0.08 | 4.9 ± 0.08 | 7.1 ± 0.18 | 11.9 ± 0.18 | 17.5 ± 0.30 | 22.5 ± 0.33 | 26.5 ± 0.45 |

Ten and 100 minute irradiation at 2,000 ergs · $cm^{-2}$ · $sec^{-1}$. Continuous light at 150 ergs · $cm^{-2}$ · $sec^{-1}$. Plants were irradiated when 4 days of age. Indicated are the standard deviations of the means.

germination in darkness at a maximum rate there is no response to gibberellic acid. However, under the laboratory conditions employed in the course of this work the maximum rate was not regularly achieved. Despite considerable effort the variable factor responsible for the observed differences has not been identified. In any case, when the growth in darkness is less than maximum, application of gibberellic acid immediately restores growth to its maximum rate. It appears, then, that this slower growth is due to a suboptimal level of natural gibberellin. Plants treated with gibberellic acid always grow at the same rate, whether irradiated or grown in darkness.

Included among the factors investigated which might be responsible for the variability in growth of plants in darkness were: Seed lot; exposure to green radiation during experimental manipulations; variations in washing and soaking procedures; water availability; and variations in temperature. The response of dark-grown Alaska pea seedlings to gibberellic acid is not increased when growth is inhibited by superoptimal or suboptimal temperatures (7). High levels of green radiation inhibit stem elongation and this inhibition may be reversed by gibberellic acid treatment ((11); Lockhart, unpublished). However, inhibition takes place only at radiation levels many times that to which these dark-grown plants are exposed. Inhibition caused by restricting the availability of water was accomplished by germinating pots of seeds in various concentrations of mannitol. Gibberellic acid treatment did not reverse the inhibition caused by high (e.g., 0.1 to 0.3 M) osmotic concentrations of mannitol. No gibberellic acid response could be related to any of the other variables outlined above.

For these reasons, then, the gibberellic acid-treated plants are taken as the dark controls when the difference between dark-grown treated and untreated plants is significant. This makes the interpretations more clear-cut but is not essential for the conclusions reached here.

TABLE II

EFFECT OF CONTINUOUS FAR-RED RADIATION ON THE GROWTH OF ALASKA PEA SEEDLINGS, AND THE REVERSAL OF FAR-RED INHIBITION BY GIBBERELLIC ACID TREATMENT

| RADIATION | GA | HEIGHT IN CM |
|---|---|---|
| Dark | control | 16.2 ± 0.34 |
| Dark | 1 $\mu$g | 19.2 ± 0.70 |
| Far-red* | control | 13.5 ± 0.60 |
| Far-red | 1 $\mu$g | 19.6 ± 0.58 |

Plants 4 days old at time of treatment. Measurements 4 days later. Gibberellic acid was applied at the time irradiation was begun. The standard deviations of the means are indicated.

*Far-red intensity ca. 450 ergs · $cm^{-2}$ · $sec^{-1}$ at 730 m$\mu$.

## DISCUSSION

Previous studies on the action spectrum of stem growth inhibition in Pisum have always utilized dwarf varieties, since the dwarfs are much more sensitive to light (9, 12). It was found in the earlier studies that red radiation was most effective in causing growth inhibition. The present author has confirmed the effectiveness of red on a dwarf variety, and found that the effect of the red radiation was reversed by subsequent far-red irradiation, as would have been expected.

The effects of visible radiation on Alaska pea seedlings, as reported here, are difficult to relate to the known morphogenetic effects of visible radiation. With the Alaska pea, there is little or no effect of the low energy red : far-red pigment system on stem elongation. The red : far-red system is present in the plant, however, and exerts effects on node formation and leaf development (12).

The distinction, reported here, between low intensity and high intensity radiation effects cannot be compared to the low energy and high energy responses already known. Both radiation effects reported here must tentatively be included with the high energy responses. Not only do these effects require a long exposure to light (if not high intensity), but it also appears that the effect of blue radiation is as great as that of the red, typical of the action spectra of Mohr (8) and of Siegelman and Hendricks (10). Inhibition by far-red radiation (and reversal of this inhibition by gibberellic acid) also is consistent with the action spectrum reported for the high energy effects. While it may be seen that the total energy required for maximum low intensity inhibition (1.0 ergs · $cm^{-2}$ · $sec^{-1}$ × 60 × 60 × 24 = 86,400 ergs · $cm^{-2}$) is much less than the energy used in the brief high intensity irradiation (2,000 ergs · $cm^{-2}$ · $sec^{-1}$ × 60 × 100 = 1.2 × $10^7$ ergs), it has repeatedly been reported in the past that reciprocity does not hold for irradiation effects on etiolated plants. Responses elicited through the red : far-red pigment in other species require of the order of 20,000 ergs · $cm^{-2}$ for a 50 % response.

It appears clear that inhibition of stem elongation in Pisum by visible radiation is mediated through the natural gibberellin system of the plant. Radiation apparently makes the natural gibberellin unavailable for growth. In the Alaska—and perhaps in other climbing varieties— after 1 to 2 days of irradiation, the metabolism changes so that the gibberellin is no longer limiting growth. It is not clear whether the level of gibberellin is restored to the dark level or whether the requirement for gibberellin is less in the irradiated plants. In dwarf peas (Morse's Progress # 9) where the sensitivity to applied gibberellic acid may be compared in light and darkness, no difference in sensitivity is found (Lockhart, unpublished).

Whatever the mechanism of recovery after irradiation, at higher intensities the recovery is not complete. A new steady-state growth is established at less than the maximum growth rate.

Björn and Virgin (1) have recently published a report on the effects of daily exposures to red light on *Pisum sativum*, variety Svalöfs tordagsärt III, apparently a non-dwarf variety. They found in many cases that plants which received daily irradiations were as tall or taller than dark-grown plants at the end of the experiment. The time-course of growth was not considered by these workers, but it is possible to determine the time-course of a single experiment from the data presented (see their fig 2). In this case an initial inhibition of stem growth by light may be observed, followed by a recovery as reported here. The irradiated plants in this case were virtually the same height as the dark controls at the end of the experiment (their usual time of measurement). The time course of growth makes it clear, however, that this is due to a gradual decline in growth rate of the dark-grown plants, while, following the initial inhibition the irradiated plants resumed a normal growth rate. A comparable decline in growth rate of the irradiated plants, presumably due to exhaustion of growth substrates, had only begun at the termination of the experiment. Probably the final height of the 2 groups would be very nearly the same. The rate of node formation, the criterion used by these authors was, of course, markedly affected by light.

Idle (3) has investigated the morphological region of maximum light sensitivity in *Vicia faba*. Using light of very brief duration, he found that when the elongating region of the stem was illuminated growth inhibition was observed but recovery soon occurred. When the tip was illuminated inhibition of growth also occurred but in this case the inhibition persisted for a much longer time (several hours). These results are consistent with the report that the natural gibberellin is produced in the stem tip (5), and the theory that light affects growth by reducing the available gibberellin in the plant. It would be expected, then, that irradiation of the elongating region would affect only the relatively small amount of gibberellin presumably present at any given time, while irradiation of the tip (the source of the gibberellin) should have longer lasting effects.

## Summary

The time-course of the stem growth of *Pisum sativum*, variety Alaska, to light and to gibberellic acid has been measured.

Under relatively low intensity red or blue light (0.1 to 100 ergs · $cm^{-2}$ · $sec^{-1}$) the growth of Alaska pea seedlings is markedly inhibited for 24 to 36 hours. Following this inhibition the plants resume a maximum rate of stem elongation (equal to dark-grown plants) in spite of the continued irradiation.

At higher radiation intensities (>100 ergs · $cm^{-2}$ · $sec^{-1}$) the plants show the same inhibition and recovery but subsequent growth is somewhat less than the maximum (dark-grown) rate, and is a function of the light intensity.

Gibberellic acid reverses both the low intensity and high intensity light inhibition of stem elongation. The low energy red : far-red pigment system does not appear to affect stem elongation in this plant.

The gibberellic acid used here was obtained through the courtesy of Merck and Company, Rahway, New Jersey and Eli Lilly and Company, Indianapolis, Indiana.

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# ENDOGENOUS SUBSTRATES OF DORMANT, ACTIVATED AND GERMINATING ASCOSPORES OF NEUROSPORA TETRASPERMA[1, 2]

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The breaking of the dormancy of Neurospora ascospores results in a 20- to 30-fold increase in their respiratory rate (5, 6). Germination can be induced by means of a heat-shock at 60° C for 10 to 30 minutes as described by Shear and Dodge (17) and Goddard (5). In addition, treatment with very low concentrations of furfural (2) and related heterocyclics (22) have also proven to be effective, as have high concentrations of aliphatic alcohols, esters and ketones (26).

Two general types of explanation of the activation process have been offered, including those relating the effect to changes in permeability and those which propose a metabolic explanation. A change in permeability is probably not directly responsible for accomplishing the activation of these ascospores because they do not require exogenous nutrients (23) nor do they respond to increased concentrations of oxygen (6). Furthermore, unpublished experiments in this laboratory have suggested that permeability to water is not a factor so that our attention has been concentrated upon an explanation based upon a metabolic control mechanism.

As a preliminary to an investigation of the enzymatic changes which ensue upon activation, a study of the substrates which are used during this time was undertaken. Previous work has shown that substrates provided exogenously are not utilized by dormant and newly activated ascospores (23, 25) so that energy sources of endogenous origin were investigated.

## MATERIALS AND METHODS

HARVESTING AND PREPARATION OF ASCOSPORES: Ascospores of *Neurospora tetrasperma* were obtained by crossing strains 377.4 and 377.5, originally isolated by Dr. B. O. Dodge, and grown and harvested by the techniques of Goddard (5). Contaminant conidia and mycelial fragments were removed by suspending the freshly harvested ascospores in 1 liter of distilled water, in a measuring cylinder, to allow the ascospores to settle over night at 4° C. By repeating this sedimentation about 5 times very clean suspensions of ascospores were obtained. Then, ascospores were suspended in 0.8 % ethylene diamine tetraacetic acid (Versene), shaken for 24 hours and cleaned in several changes of distilled water, as described by Sussman (23). Such treatment has no effect on dormant ascospores whereas any remaining vegetative cells or conidia are killed.

Air-drying was accomplished by using a continuous flow of dry, filtered air for about 24 hours with occasional turning. Such air-dried spores were found to contain 4 to 5 % moisture, as determined by the loss of weight on oven-drying at 102° C for 24 hours. The air-dried ascospores were stored in screw cap bottles at 4° C and none were used in the following experiments that had been harvested longer than 3 months previously, unless mentioned otherwise. Each experiment was carried out in triplicate and samples usually consisted of 52.5 mg of ascospores (50 mg dry weight). The terminology of the different physiological stages of the organism, including the dormant, activated, germinating and vegetative stages, are as suggested by Goddard (6) and extended by Sussman et al (24).

Dormancy of ascospores was broken by exposure to 60° C for 20 minutes and incubation was accomplished on a rotary shaker in distilled water at 20° or 26° C, for 3 to 4 hours. Before incubation for extended periods of time, dormant ascospores were surface-sterilized with 0.5 % commercial Clorox for 3 minutes, rinsed with sterile distilled water several times and incubated dry or in distilled water on a rotary shaker for several weeks or months.

KILLING AND CRUSHING OF ASCOSPORES: Killing was accomplished by boiling for 3 minutes in 75 % alcohol (84° C), after which the spores were centrifuged and the supernatant alcohol was saved for carbohydrate analysis. Lipids were not extracted by such treatment but the supernatant did contain an-

[1] Received December 12, 1958.

[2] This work was greatly aided by grants from the Michigan Memorial Phoenix Project of the University of Michigan, and from the National Science Foundation.

throne-positive materials. In order to determine the amount of carbohydrates that were extractable by this means, dormant and activated ascospores were killed and the amount of anthrone-positive materials in the supernatant was analyzed. It is evident from the data in table I that the amount of such materials was reduced by more than one-half when spores cleaned by the means described above were used. Moreover, activation does not increase the amount of carbohydrate released except after 3 hours of incubation. Similar results were obtained when ascospores were incubated alive in water so that killing alone does not result in greater leakage of carbohydrates from these cells. There is an increase in the amount of carbohydrate released when the cells are incubated for 30 hours in 80 % alcohol after killing. This increase is maximal when cells are incubated in water for at least 3 hours before being killed. Thus, after all possible precautions were taken to remove contaminating materials from ascospore suspensions, 0.3 to 0.4 mg of anthrone-positive materials still appeared after killing so that this amount was included in the 80 % alcohol-soluble fraction.

The killed ascospores, recovered after centrifugation, were thoroughly mixed with about 4 times their volume of glass beads (Minnesota Mining & Manufacturing Co., Super-Brite pavement markers), and placed in Nossal capsules, cooled in an ice bath, and crushed by agitation in a Nossal disintegrator (14). The capsules were shaken for 8 periods of 20 seconds duration, between each of which the capsules were cooled in brine in order to minimize heating during the crushing of the ascospores. Under these conditions, visual examination revealed that the spores are completely disintegrated and form a paste around the glass beads.

Lipid Extraction and Analysis: The contents of the Nossal capsule were transferred into 15 ml of a chloroform-methanol mixture (2 : 1 by volume), kept at room temperature for 5 hours with occasional shaking, after which the supernatant was recovered after centrifugation. The sediment was reextracted with 15 ml of solvent for 3 periods of 5 hours each, and a single extraction over night. The last 2 extractions were made at 45° C and the first 2 at room temperature. The entire lipid extract was shaken with an equal volume of deionized water and kept over night at 4° C (21). The upper (water-methanol) phase was separated, made up to volume and saved for carbohydrate analysis. The lower, chloroform phase, was vacuum-dried, redissolved in petroleum ether (35° C boiling point) and, in turn, vacuum-dried. This material was dissolved in 50 ml petroleum ether and aliquots were used for qualitative and quantitative analysis. Esters were determined colorimetrically by the method of Goddu et al (7), using tripalmitin as a standard. Total lipid phosphorus was determined by the method of Fiske and Subbarow (4), and the occurrence of free fatty acids was investigated by adding excess diazomethane and estimating the increase in esters by the methods described above. Plasmalogens were analyzed for by the fuchsin-aldehyde reaction described by Shriner and Fuson (18). Ergosterol was determined colorimetrically by the method of Sobel et al (20), using 4 % acetyl chloride in glycerol 1,3-dichlorhydrin, with a solvent : reagent ratio of 1 : 4, and ergosterol as a standard.

Carbohydrate Extraction and Analysis: Defatted, crushed ascospores that had been prepared as described above, were extracted 3 times, during a 12-hour period, with 80 % ethanol at 60 ° C (1), using 4 volumes of 80 % ethanol. A 4th extraction in this solvent was carried out over night at the same temperature. The clear supernatant was recovered after centrifugation, made up to volume, and saved for further analysis. The polysaccharides remaining in

Table I

Extraction of Carbohydrates from whole Ascospores of Neurospora tetrasperma

| Treatment | Preparative technique | | |
|---|---|---|---|
| | Water* | Prolonged extraction** | Short extraction† |
| | *mg carbohydrate per 100 mg ascospores* | | |
| Dormant (clean)†† | 0.2 (15 hrs) | 0.53 | 0.27 |
| Dormant (clean)†† | 0.3 (30 hrs) | ... | ... |
| Dormant (not cleaned) | 0.6 (6 hrs) | 0.8 | 0.6 |
| Activated,†† incubated: | | | |
| 0 hrs | 0.3 (30 min) | 0.48 | 0.3 |
| 1.5 hrs | 0.3 (2 hrs) | 0.4 | 0.3 |
| 3 hrs | 0.3 (3.5 hrs) | 1.13 | 0.4 |
| 5 hrs | 0.4 (5.5 hrs) | 1.2 | 0.4 |
| 12 hrs | 0.4 (12.5 hrs) | 1.5 | 0.6 |

Carbohydrates were determined on the basis of dry weight of ascospores. Spores were heat-activated at 60° C and incubated by shaking at 20° C.

* The figures in parentheses indicate the amount of time in the extraction medium which was held at 20° C.

** Ascospores were killed by boiling for 3 minutes in 80 % ethanol, thereafter extracted in the same medium for 30 hours at 60° C.

† Killed by boiling in alcohol for 3 minutes.

†† Cleaning was performed by sedimentation and washing as described in the text.

the ascospores that had been extracted in 80 % alcohol were extracted in 1 volume of 72 % sulfuric acid at 4° C (9, 12). Four extractions were made during a 48-hour period, after which the supernatant was diluted to 100 ml with water. The residual spore material was shown not to contain any anthrone-positive material. Total carbohydrates were estimated by the anthrone method described by Morris (13), using glucose as the standard and filter no. 54 in the Klett colorimeter. Reducing sugars were estimated by Nelson's method described by Bell (1), and glucose by the use of glucose oxidase (Glucostat reagent, Worthington Biochemical Corp., Freehold, N. J.) and a chromogenic hydrogen donor in the presence of peroxidase in order to form a colored product (16).

## Results

Substrate Utilization in Dormant Ascospores: A low rate of metabolism is maintained by dormant ascospores ($Q_{O_2} = 0.5$) relative to that of activated ones ($Q_{O_2} = 20$) (5, 6). Therefore, it is to be expected that the rate at which substrate disappears in the former case will be correspondingly slower. For this reason, long-term experiments were necessary in order to detect the decrease in these materials. Ascospores that had been stored for 2 years at 4° C were analyzed for their content of lipids and carbohydrates. In addition, those that were a month old were incubated by shaking in water at 21° C and samples were analyzed in the same way. The results outlined in table II reveal that lipids disappear from dormant ascospores during prolonged incubation, even at 4° C, whereas carbohydrates are utilized much more slowly or not at all. That the spores were completely germinable throughout the incubation period was shown by activating a small aliquot of the spores at the time analyses were made, after which they were incubated and the percentage of germination determined.

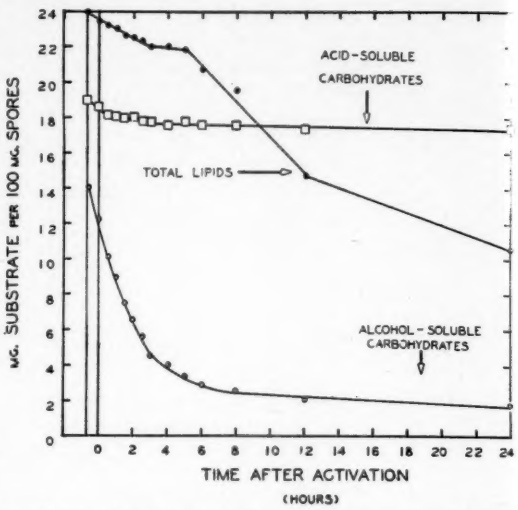


Fig. 1. Effect of activation upon the amount of lipids and carbohydrates in ascospores of *Neurospora tetrasperma*. Ascospores were heat-activated and germination began about 2.5 hours after activation and was complete about 4 hours after. Determinations were performed as described in the text.

Activation and Lipid Metabolism: Ascospores that were 1 month old were activated and incubated for varying lengths of time after which aliquots were withdrawn and analyzed for their lipid content. These results are provided in figure 1 and disclose that there is a small change in the amount of these materials during the 1st 4 to 5 hours after activation. Beyond this time, there is an acceleration in the use of this substrate until after 12 hours when it is somewhat decreased. It should be noted that germination is completed between 3 to 4 hours after

Table II

Changes in the Endogenous Substrates of Dormant Ascospores of Neurospora tetrasperma

| Age of ascospores | Ethanol-soluble carbohydrates | Acid-soluble carbohydrates | Total lipids | Germinability % |
|---|---|---|---|---|
| | *mg* | *mg* | *mg* | |
| Two-years-old (harvested 1956 and stored at 4°C in air) | 13.6 | 19 | 21.4 | 82 |
| One-month-old (harvested 1958 and stored at 4°C in air) | 14 | 19 | 24 | 94 |
| One-month-old (stored at 4° C then shaken 15 days in water at 21° C) | 14 | 19 | 23.4 | 93 |
| One-month-old (stored at 4° C then shaken 40 days in water at 21° C) | 14 | ... | 21 | 96 |

All values are expressed per 100 mg ascospores (dry weight).

TABLE III

LIPIDS OF DORMANT AND ACTIVATED ASCOSPORES OF NEUROSPORA TETRASPERMA

| TIME AFTER ACTIVATION | TOTAL LIPIDS | TOTAL ESTERS* | PHOSPHOLIPIDS** | STEROLS*** |
|---|---|---|---|---|
| *hrs* | *mg* | μM | μM | μM |
| Dormant | 26.6 | 86.7 | 5.36 | 11.24 |
| 0 | 25.5 | 91.9 | 6.04 | 12.23 |
| 0.5 | 24.5 | 98.7 | 5.28 | 12.05 |
| 1 | 25.7 | 96.7 | 5.05 | 10.59 |
| 1.5 | 24.8 | 102.4 | 5.12 | 10.08 |
| 2.5 | 23.9 | 96.4 | 6.08 | 10.08 |
| 3.5 | 25.0 | 102.1 | 7.18 | 9.33 |
| 7.5 | 21.7 | 96.8 | 11.97 | 9.83 |

All values based upon 100 mg ascospores (dry wt).

* Calculated as tripalmitin.

** Calculated on the basis that 1 M of inorganic phosphate is equivalent to 1 M of lecithin.

*** Calculated as ergosterol.

activation so that the maximum rate of utilization of lipids occurs only after this is accomplished.

A more detailed examination of the lipids of ascospores was made through analysis of the ester, phospholipid and sterol fractions of their lipid contents. As table III discloses, a slight increase in esters was detected within 0.5 hour after activation but, thereafter, there was very little change. Similar results were found for the phospholipids except that after germination, an increase was found to occur. As for the sterol fraction, which was analyzed with ergosterol as the standard, a small decrease was observable after 1 hour. In this connection, it was found that, when the method of Sobel et al (20) was applied to this fraction, it turned greenish within a minute after the addition of the reagent. On the other hand, ergosterol became pink first and turned fluorescent-green gradually so that it is possible that calciferol occurs in the ascospores, or is produced from ergosterol during storage in ether. Qualitative tests of the lipids were also performed with the results indicated in table IV. Thus, the presence of ergosterol, or calciferol, was indicated by 2 tests but no cholesterol could be found. Plasmalogens, a distinct group of acetal phosphatides, have been isolated from animals and green plants (27). Their absence in ascospores (table IV) suggests that the phosphate in the lipid materials of these cells is present mainly in phospholipids. The absence of methyl esters, after treatment with the strong methylating agent diazomethane, suggests that free fatty acids are not present in lipid extracts of spores, or are in concentrations too low to be detected.

TABLE IV

QUALITATIVE ANALYSIS OF LIPIDS OF NEUROSPORA ASCOSPORES

| TEST | SUBSTANCES ANALYZED | RESULTS |
|---|---|---|
| Diazomethane | methyl-ester formation | — |
| Fuchsin-aldehyde reagent (Shriner and Fuson, 1948) | plasmalogen | — |
| Lieberman-Burchard reagent | cholesterol | — |
| Salkowski's reagent | ergosterol | + |
| Acetyl chloride (Sobel et al, 1945) | ergosterol (calciferol) | + |

Lipid extracts of all the spore samples listed in table III were analyzed separately. In all cases identical results were obtained so that composite results are presented in the table above.

ACTIVATION AND CARBOHYDRATE METABOLISM: The possibility that carbohydrates were utilized as an energy source was also investigated. Ascospores that were 1 month old were incubated for various times after activation and the ethanol-soluble, acid-soluble and total carbohydrates were analyzed. These results are presented in table V and figure 1 and reveal that a striking decrease in the ethanol-soluble carbohydrate fraction occurs immediately following activation. As a matter of fact, a perceptible change occurs during the period of heat-treatment at 60° C. By contrast, the content of acid-soluble carbohydrates remains largely unchanged during the germination period and as long as 24 hours after activation.

TABLE V

CARBOHYDRATE FRACTIONS OF DORMANT AND ACTIVATED ASCOSPORES OF NEUROSPORA TETRASPERMA

| TIME AFTER ACTIVATION | ETHANOL-SOLUBLE CARBOHYDRATES | ACID-SOLUBLE CARBOHYDRATES | TOTAL CARBOHYDRATES |
|---|---|---|---|
| *hrs* | *mg* | *mg* | *mg* |
| Dormant | 14.0 | 19.4 | 33.4 |
| 0 | 12.1 | 18.6 | 30.7 |
| 0.5 | 10.4 | 18.2 | 28.6 |
| 1 | 8.9 | 18.1 | 27.0 |
| 1.5 | 7.4 | 18.0 | 25.4 |
| 2 | 6.5 | 18.1 | 24.6 |
| 2.5 | 5.6 | 17.8 | 23.4 |
| 3 | 4.4 | 17.8 | 22.2 |
| 4 | 4.0 | 17.6 | 21.6 |
| 5 | 3.3 | 17.8 | 21.1 |
| 6 | 2.8 | 17.6 | 20.4 |
| 8 | 2.6 | 17.6 | 20.2 |
| 12 | 2.1 | 17.4 | 19.5 |
| 24 | 1.9 | 17.5 | 19.4 |

All values expressed per 100 mg ascospores (dry wt).

The effect of heat-activation upon the soluble carbohydrates was studied in more detail by maintaining ascospores at 60° C for up to 4 hours. Aliquots were withdrawn at intervals during this time and the carbohydrates analyzed. Separate aliquots were incubated at 26° C for 3 hours and the percentage of germination determined. The rate of disappearance of carbohydrates is greatest up to 30 minutes after the start of incubation and rapidly decreases after 1 hour (table VI). Germination will not occur if incubation is continued at the activation

TABLE VI

EFFECT OF CONTINUED EXPOSURE TO 60° C ON CARBOHYDRATES OF ASCOSPORES OF NEUROSPORA TETRASPERMA

| DURATION OF INCUBATION | ETHANOL-SOLUBLE CARBOHYDRATES | ACID-SOLUBLE CARBOHYDRATES | GERMINATION |
|---|---|---|---|
| | *mg* | *mg* | % |
| Dormant | | | |
| control | 14.1 | 19.4 | 0 |
| 5 min | 14.1 | ... | ... |
| 10 " | 14.0 | ... | ... |
| 15 " | 13.3 | ... | ... |
| 20 " | 12.0 | ... | 93 |
| 30 " | 11.1 | ... | ... |
| 1 hr | 10.7 | ... | ... |
| 1.5 hrs | 9.9 | ... | ... |
| 2 " | 9.3 | ... | ... |
| 2.5 " | 9.3 | ... | ... |
| 3 " | 8.8 | ... | ... |
| 4 " | 8.3 | 18.2 | 88 |

Amount of carbohydrates expressed per 100 mg ascospores (dry wt).

temperature but if the ascospores are removed to 27° C, even after 4 hours at 60° C, a high percentage of germination is obtained.

Because of the effect of heat-activation upon the ethanol-soluble carbohydrates, the composition of this fraction was investigated. In these experiments, the procedure was modified from that used above in that only 3 extractions were carried out instead of the 4 used in the preceding experiments and it will be observed that the amount of carbohydrates extracted is somewhat lower than before. The dry matter, as well as the anthrone-positive materials in the alcohol-soluble fraction were investigated with the results shown in table VII. Although there are materials other than carbohydrates which are extracted, they remain relatively constant in amount until 6 hours after activation when a decrease occurs. Both reducing sugar and glucose determinations were performed on this fraction and the results summarized in table VIII. It is at once apparent from these data

TABLE VII

CHANGES IN DRY MATTER AND CARBOHYDRATES IN THE 80 % ETHANOL-SOLUBLE FRACTION OF DORMANT AND ACTIVATED ASCOSPORES OF NEUROSPORA TETRASPERMA

| TIME AFTER ACTIVATION | DRY MATTER | TOTAL CARBOHYDRATES (a) | NON-CARB. SOLIDS (b) | a / b |
|---|---|---|---|---|
| *hrs* | *mg* | *mg* | *mg* | |
| Dormant | 17 | 11.3 | 5.7 | 2.0 |
| 0 | 15 | 10.0 | 5.0 | 2.0 |
| 1 | 12 | 4.3 | 7.7 | 0.6 |
| 2 | 10 | 2.7 | 7.3 | 0.4 |
| 3 | 8 | 1.9 | 6.1 | 0.3 |
| 6 | 4 | 1.1 | 2.9 | 0.4 |

Carbohydrates were determined with the anthrone reagent and all values are calculated on the basis of 100 mg of ascospores (dry wt).

that only a small fraction of the anthrone-positive material is reducing sugar, most of which is glucose.

## DISCUSSION

As judged by their gradual disappearance from dormant ascospores of *Neurospora tetrasperma*, lipids are the substrate which is utilized by this organism during this stage in its life cycle. By extrapolation, and assuming that fat is the only fuel that can be utilized by dormant cells, sufficient substrate is present for spores to survive about 18 years at 4° C, but only for 320 days when shaken at 21° C in water. By contrast, however, carbohydrates disappeared much more slowly from dormant spores; in fact, this decrease was imperceptible in the 40 days during which the analyses were performed. While it might be argued that the life of dormant cells could be extended by the provision of exogenous substrates, the failure of any but the smallest molecules to penetrate largely precludes this possibility (11, 25).

Upon activation the ethanol-soluble carbohydrate pool is rapidly utilized and is the major substrate

TABLE VIII

REDUCING SUGAR AND GLUCOSE CONTENT OF THE 80 % ETHANOL-SOLUBLE FRACTION OF DORMANT AND ACTIVATED ASCOSPORES OF NEUROSPORA TETRASPERMA

| TIME AFTER ACTIVATION | TOTAL CARBOHYDRATE | REDUCING SUGAR | GLUCOSE |
|---|---|---|---|
| *hrs* | *mg* | *mg* | *mg* |
| Dormant | 10.67 | 0.04 | 0.05 |
| 0 | 9.80 | 0.03 | 0.02 |
| 1 | 4.67 | 0.06 | 0.06 |
| 2 | 2.47 | 0.05 | 0.04 |
| 3 | 2.07 | 0.02 | 0.02 |
| 6 | 1.07 | 0.02 | 0.01 |

Extracts were prepared by evaporation of the ethanol, after which the residue was redissolved in distilled water. All values are based upon 100 mg of ascospores (dry wt).

until the protrusion of the germ tube. Even after a few minutes of activation by exposure to 60° C, a significant decrease in this fraction can be noted. Reducing sugars comprise only a small fraction of the soluble carbohydrates, most of which is probably glucose. Lipids are also utilized at a faster rate after activation so that 2 substrates disappear during this time. After the protrusion of the germ tube (3 to 4 hours after activation), and at a time corresponding to that when the soluble carbohydrate pool is almost completely depleted, another increase in fat consumption occurs which is maintained until 12 hours after activation. Beyond this time there is a diminished rate of disappearance of these lipids, at a level corresponding to that during the 1st 3 or 4 hours after activation. These data support and extend the previous work by Goddard (6) and Holton (8) which has shown that the respiratory quotient (R.Q.) of dor-

mant and germinating ascospores is 0.6 whereas that of activated ones varies, but is usually closer to 1.0 than at any other time in the development of these cells.

That lipids serve as an important reserve material in many other fungus spores was suggested by Kordes (10) who showed that there was coalescence of fat drops when germination occurred. However, his observations were largely qualitative in that they were based upon the appearance of the fat droplets during the various stages in the life cycle. On the other hand, it is fairly well established in uredospores of *Puccinia graminis* (19) that the saponifiable fraction of the lipids is reduced during germination in parallel with the situation in many seeds. By contrast, Ergle (3) has shown that glycogen is the substrate utilized by sclerotia of Phymatotrichum during their germination so that this case, taken together with that of Neurospora ascospores, suggests that the propagules of fungi may utilize endogenous substrates other than lipids.

These results also bear upon the question of whether activation induces a qualitative, as well as quantitative, change in the metabolism of these ascospores. Goddard (6) first proposed that pyruvate carboxylase activity was induced as a result of the disruption of dormancy. He came to this conclusion on the basis that the inhibition by fluoride of the respiration of activated cells was reversed by pyruvate, whereas that of dormant cells was not. Moreover, he established that pyruvate was absorbed so that the effect could not be ascribed to a difference in permeability. Recently, however, it was found (24) that the pyruvate carboxylase and cocarboxylase activity of ascospores is not markedly altered as a result of activation so that another explanation must be sought. The sequence of metabolic events which ensues upon germination, as described by Sussman, Distler and Krakow (24), is as follows: 1) Immediately upon the termination of dormancy, fermentation products like ethanol, acetaldehyde and pyruvate accumulate. However, shortly before germination their production is curtailed and their concentration levels off; and 2) the full complement of Krebs cycle acids does not appear until shortly before germination, or simultaneously with the limiting of the production of fermentation products. These authors suggested that activation evokes a fermentative system, possibly of the Embden-Meyerhof-Parnas type. Shortly before germination, possibly by means of a sequential induction, an oxidative type of metabolism arises which may function through the tricarboxylic acid cycle, or a variant, thereof. The present results support the idea that a qualitative change in the metabolism of ascospores of Neurospora does occur and, moreover, show that the locus of the effect may be in the pathways of carbohydrate dissimilation.

These data permit a comparison to be made between some of the chemical constituents of ascospores and conidia of Neurospora. Such a comparison is an approach to an understanding of differentiation insofar as the functions of these constituents are understood. The information in table IX reveals that the major difference between these types of spores is in the lipid fraction. Thus, ascospores contain larger amounts of esters than do conidia whereas the reverse is true in the case of phospholipids. Moreover, the soluble carbohydrate fraction is larger in ascospores than in conidia but the reverse is true of the insoluble fraction. The meaning of these data is difficult to assess but the surprising discrepancy in the content of phospholipids suggests that structural differences, perhaps in membranes, may be concerned.

TABLE IX

COMPARISON OF SOME OF THE CONSTITUENTS OF CONIDIA AND ASCOSPORES OF NEUROSPORA

| | PERCENTAGE COMPOSITION | |
|---|---|---|
| | DORMANT ASCOSPORES* | CONIDIA** |
| Lipids (total) | 26.6 | 17.7 |
| Sterols | 1.3 | 0.5 |
| Esters | 19.3 | 0.1 |
| Phospholipids | 1.1 | 13.8 |
| Carotenoids, fatty acids | 4.9*** | 3.3 |
| Carbohydrates (total) | 33.0 | 36.5 |
| Soluble (in alcohol or hot water) | 14.0 | 9.3 |
| Insoluble | 19.0 | 27.2 |
| Water content (air dried) | 5.0 | 9.1 |

The figures are calculated on the basis of the dry weight of the cells.

* Data from this paper, on *Neurospora tetrasperma*.

** Data of Owens et al (1958) on *Neurospora sitophila*.

*** By difference.

## SUMMARY

Ascospores of *Neurospora tetrasperma* have been shown to contain about 25 % total lipids and 33 % carbohydrates. The former serve as the source of energy for dormant ascospores which have enough of this substrate to survive for 18 years when stored at 4° C, or for less than 1 year when shaken in water at room temperature. On the other hand, carbohydrates do not appear to be utilized during dormancy but begin to disappear almost immediately after the spores are heat-activated. Fats also are utilized during this time, but in lesser amounts than carbohydrates. Only those carbohydrates soluble in hot 80 % ethanol are affected in this way for the acid-soluble fraction remains almost constant in amount, after a small initial decrease upon activation. Reducing sugars and glucose comprise less than 1 % of the ethanol-soluble carbohydrates, while esters and sterols are the major constituents of the lipids. A comparison is made between conidia and ascospores of Neurospora on the basis of their content of lipids and carbohydrates.

The authors would like to thank Drs. Saul Roseman, Edward Heath, and William Lands, and Mr. Jack Distler for helping in the design and evaluation of techniques used in these investigations.

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# INFLUENCE OF TEMPERATURE ON THE RESPONSE OF GERMINATING BARLEY GRAINS TO POTASSIUM GIBBERELLATE [1,2,3]

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Since their discovery the gibberellins have been found to influence a number of plant processes in addition to promoting the growth of stems. The significance of the gibberellins as plant growth regulators has increased, especially since the evidence is now strong that they may occur naturally in higher plants (7, 12, 13).

Gibberellins have been found to replace vernalization requirements (4, 17), and to substitute for day length requirement (17). Gibberellins have promoted the flowering of plants and have initiated fruit set without pollination (18). They are known to break the tuber dormancy of potatoes (14) and several species of grasses have been induced to grow out of season by application of gibberellins (5).

Gibberellins can overcome the light inhibition of etiolated pea seedlings (6), and can substitute for the red light requirement of germinating lettuce seeds (3).

In a study of the effect of temperature on the response of Alaska pea to gibberellic acid, Lockhart concluded that gibberellic acid was stimulatory at all temperatures (8).

In this laboratory a program is underway to screen radiation induced mutants of the fungus *Gibberella fujikuroi* for their production of gibberellins. To that end germinating barley grains were investigated for use as a bioassay material. In the course of numerous trial runs to establish the variability of response of the germinating barley grains to various concentrations of potassium gibberellate, a temperature effect was noted which results in inhibition of growth at low concentrations of potassium gibberellate at moderately low temperatures. In order to verify this phenomenon additional experiments were initiated which culminated in this report.

## METHODS AND MATERIALS

Grains of barley, variety Montcalm, were steeped in water for 24 hours at 18° C. They were then transferred to Petri dishes and allowed to partially germinate on water moistened filter paper for an additional 48 hours, also at 18° C. The 18° C temperature was chosen as it approximates the malting temperature. The lengthy period of partial germination prior to selection and treatment was used to insure uniformly germinating barley grains for testing. Those barley grains which had 3 seminal roots about 1 cm long were selected for treatment in various concentrations of potassium gibberellate at 3 temperatures.

Grains in lots of 15 were selected for each treatment. The treatments consisted of 5 concentrations of potassium gibberellate, and distilled de-ionized water. Identically treated sets of barley grains were prepared by placing them in concentrations of 0.001, 0.01, 0.1, 1, and 10 ppm potassium gibberellate (82 % pure supplied by Merck and Company). Distilled water was used for the check. In all cases the lots of 15 grains were placed in Petri dishes on filter paper moistened with 5 ml of the test solution. One set of 6 dishes was placed in each of the following temperatures, 15, 18, and 25° C.

Each set was placed in the 15, 18, or 25° C for 48 hours after which all sets were placed at 25° C for an additional 24 hours. The grains were placed at 25° C for 24 hours to make certain that all acrospores of the grains grew and to make measurement easier. Measurements were made of the lengths of the primary leaves, coleoptiles, and the longest root at this time. Seedlings were held in a 3° C refrigerator until measured. No seedlings were held more than 2 hours and it is assumed that no major changes occurred in the seedlings in that time. Since refrigerators were used exclusively to obtain the temperatures for the experiments, the seedlings grew in darkness except for the time of the selection of the partially germinated grains for testing and at that time only the seminal roots of the germinating grains had emerged. At the time of measurement the seedlings were chlorotic. The experiment was repeated 3 times.

## RESULTS AND DISCUSSION

Figure 1 presents in summarized form the results of these experiments. It contains 3 curves for each of the measured plant parts; primary leaves, coleoptiles and roots, and the temperature and concentration conditions under which these plant parts grew.

The abscissa values for concentration are on a logarithmic scale and the ordinate values for length are on an arithmetical scale.

[1] Received January 6, 1959.

[2] Research supported by United States Atomic Energy Commission, Contract no. AT (11-1)-42.

[3] Paper no. 4055, Scientific Journal Series, Minnesota Agricultural Experiment Station.

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TABLE I

F VALUES DERIVED FROM ANALYSES OF VARIANCE FOR EACH CONCENTRATION AND PLANT PART †

| | $H_2O$ | 0.001 PPM | 0.01 PPM | 0.1 PPM | 1 PPM | 10 PPM |
|---|---|---|---|---|---|---|
| Primary leaves | 17.6*‡ | 30.8** | 14.5* | 10.4* | 28.1** | 30.3** |
| Coleoptiles | 2.2 | 15.8* | 3.2 | 2.6 | 7.3* | 2.7 |
| Roots | 8.5* | 102.6** | 11.7* | 88.6** | 103.2** | 48.6** |

† In no case were differences among replications significant.

‡ * significance at the 0.05 level; ** significance at the 0.01 level

Since the nature of the relationships indicated by the curves is not known, the points have been connected by straight lines rather than smooth curves. Analyses of variance were performed for each concentration at each temperature and the F values for these analyses are contained in table I.

PRIMARY LEAVES: The primary leaves were measured from the base of the coleoptiles to the tips of the leaves. In some cases the primary leaves had not emerged from the coleoptiles but they were plainly visible through the coleoptiles and therefore could be measured.

The data in figure 1 for primary leaves indicate that low concentration of potassium gibberellate was inhibitory at 15 and 18° C, but had no effect at 25° C. The greatest growth of primary leaves was in the range from 0.01 to 1 ppm potassium gibberellate at 25° C. This agrees with results obtained with pinto bean (1, 9), dwarf corn (11), and dwarf peas (10).

At each concentration differences among means for primary leaves were statistically significant. For distilled water, 0.01, and 0.1 ppm differences were significant at the 0.05 level and for 0.001, 1, and 10 ppm differences were significant at both the 0.05 and 0.01 level of significance.

COLEOPTILES: As with primary leaves, the concentrations 0.001 and 0.01 ppm potassium gibberellate appear to be inhibitory. The shape of the curves resembles that of the primary leaves, but not as much total growth of tissue seems to be involved. The concentration 10 ppm is inhibitory at 25° C as well as at the other 2 temperatures. In many instances the coleoptiles had already been ruptured by the primary leaves at the time of measurement. This phenomenon when it occurred seemed to occur uniformly within a treatment.

As can be seen from an examination of table I, coleoptiles are apparently more uniform in their response to temperature than the other 2 structures. Only for 2 concentrations were mean differences significant and then at the 0.05 level of significance.

ROOTS: In no case did potassium gibberellate cause an increase in the longest root over that for the distilled water controls. Whaley (16), however, reported a somewhat stimulatory effect of gibberellic acid on excised corn root tips as did Richardson (15) with roots of *Pseudotsuga menziesii* (Mirb.) Franco.

The temperature effect separates the root response into 2 magnitudes for while there are significant differences among all mean values, there were no significant differences between the curves for 15 and 18° C.

A greater inhibitory effect than might be expected is again evident for the concentrations 0.001 and 0.01 ppm and this effect is apparent at all temperatures tested. The curves for root length coincide in shape but are displaced with respect to temperature.

In order to provide a measure of the variability within treatment, standard errors were computed for all parts measured for all concentrations at all tem-

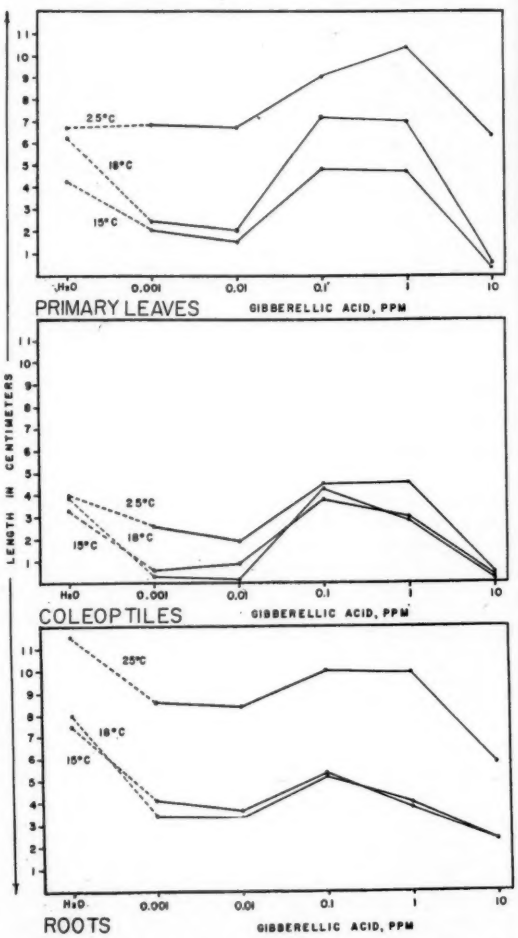


FIG. 1. Growth of primary leaves, coleoptiles, and roots of barley with respect to temperature and potassium gibberellate. Each point represents the average of 45 measurements of the plant structure indicated.

TABLE II

STANDARD ERRORS* IN CENTIMETERS COMPUTED FOR EVERY PLANT PART, TEMPERATURE AND CONCENTRATION

| | | $H_2O$ | 0.001 PPM | 0.01 PPM | 0.1 PPM | 1 PPM | 10 PPM |
|---|---|---|---|---|---|---|---|
| Primary leaves | 25° C | .42 | .68 | .61 | .87 | .92 | 1.03 |
| | 18° C | .46 | .49 | .38 | .67 | .55 | .56 |
| | 15° C | .31 | .41 | .38 | .37 | .49 | .43 |
| Coleoptiles | 25° C | .15 | .59 | .37 | .36 | .36 | .38 |
| | 18° C | .29 | .34 | .13 | .20 | .34 | .22 |
| | 15° C | .44 | .20 | .21 | .34 | .46 | .36 |
| Roots | 25° C | 1.10 | .74 | .81 | .80 | .94 | .63 |
| | 18° C | .60 | .27 | .30 | .27 | .32 | .18 |
| | 15° C | .68 | .49 | .31 | .45 | .33 | .21 |

$$*\,s\bar{x} = \frac{s}{\sqrt{n}}$$

peratures. These values are contained in table II. While these standard errors are by no means uniform and even in some cases exceed 10 % of the mean value they are not of the order of magnitude of the inhibition induced by the interaction of the lower concentrations of potassium gibberellate and the moderately low temperatures.

From the foregoing discussion a number of conclusions may be drawn. All the curves have some features in common. All show stimulation and inhibition of the growth of primary leaves, coleoptiles and roots sequentially at similar concentrations of potassium gibberellate. The range from 0.01 to 0.1 ppm in all cases is stimulatory compared to lower concentrations, but more important the range from 0.001 to 0.01 was either inhibitory in the 15 and 18° C environment or ineffective as in the 25° C temperature. This is contrary to the usual expection with plant growth substances where it is expected that low concentrations would be stimulatory and higher concentrations inhibitory. This finding is, however, in agreement with the observation of Barlow, Hancock, and Lacey (2) who demonstrated that low concentrations of indole acetic acid could be inhibitory to the growth of young wheat coleoptiles. In the experiments reported here the higher concentrations are inhibitory and under the test conditions only the intermediate concentrations seem to be stimulatory.

## SUMMARY

The growth of germinating barley grains appears to be inhibited by 0.001 and 0.01 ppm potassium gibberellate, if the germinating barley is held at moderately low temperatures. The range of concentration from 0.01 to 0.1 ppm is in general stimulatory for primary leaves and coleoptiles, however no concentration was stimulatory for roots.

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# PHOSPHORUS COMPOUNDS OF COTTON EMBRYOS AND THEIR CHANGES DURING GERMINATION [1]

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The phosphorus compounds of cotton embryos, particularly ribonucleic (RNA) and deoxyribonucleic (DNA) acid, and their metabolism during germination have not been intensively investigated with modern methods of analysis. In the period 1913 to 1918 Anderson (1) and Rather (9) elucidated the true nature of phytic acid, the principal form of seed phosphorus. Although Anderson obtained the hexaphosphate of inositol, Rather found only the pentaphosphoric acid derivative due to the activity of the enzyme phytase. In 1946 Fontaine and co-workers (5) presented results showing an increase in the percentage of phosphorus in the inorganic form during germination. Pons et al (8) developed an analytical system for analysis of the phosphorus fractions of plant materials and in 1953 reported data on the phytin-, inorganic-, lipid-, ester-, and nucleic acid-P contents of cotton embryos. More recently Ergle and Eaton (3) reported on the amounts of similar fractions, plus protein-P, in various cotton tissues including 10-day-old ovules and mature embryos.

In the present paper information on the phosphorus compounds of cotton embryos has been extended to include RNA and DNA and the changes which they and the above mentioned phosphorus fractions undergo during germination and early seedling development. Results on Paymaster 54B, Deltapine TPSA, and Mesilla Valley Acala, representative of early, intermediate, and late maturing cottons, respectively, are compared.

## MATERIALS AND METHODS

GERMINATION: Lots of 300 acid-delinted seeds of Mesilla Valley Acala, Paymaster 54B, and Deltapine TPSA cotton varieties were germinated in the dark at 28 to 30° C in moist, autoclaved vermiculite for 1, 2, and 4 days. To obtain 6-day-old seedlings, 4-day-old seedlings from the vermiculite were transferred to 2-gallon glazed jars filled with 0.005 M $CaSO_4$ solution and grown in the dark for 2 additional days with aeration. The seedlings were supported on waxed hardware cloth. Similarly, seeds of the 3 varieties were germinated and grown in sunlight in the greenhouse for harvest only at the end of the 6-day period.

HARVEST AND SAMPLE PREPARATION: The embryos from air dry seed, representing 0-day germination, were separated from the seed coats and ground in a small Wiley mill 1st to 20-mesh and later (after being defatted) to 80-mesh fineness before analysis. The seedlings, after removal of vermiculite and any adhering seed coats, were lyophilized and ground to 80-mesh fineness. The cotyledons were separated mechanically from the remainder of the seedling and analyzed separately in most of the experiments.

ANALYSIS: The analytical methods used in the separation of (a) lipid-, phytin-, inorganic-, and ester-P and (b) RNA- and DNA-P were basically those described by Pons et al (8) and Ogur and Rosen (7), respectively. However, since the 2 methods were combined into a single analytical system (plus some modification) in the present work, the procedure used is briefly described. Except when noted, the phosphorus contents of the separated fractions were determined after wet ashing with a minimum amount of $H_2SO_4$ by the method of Fiske and Subbarow (4).

*Lipid-P*: Five grams of embryos or 3 g of the germinated tissue were extracted at a rapid rate for 8 hours each time in a Soxhlet apparatus; 1st with petroleum ether (B.P. 30 to 65° C) and then with an alcohol : benzene azeotrope (32.4 % : 67.6 %, by weight, B.P. 68.2° C). The phosphorus contents of the evaporated extracts were determined separately.

*Phytin-P*: Phytin was extracted from a 1 g sample of the lipid-free residue above with a 2 % HCl–10 % $Na_2SO_4$ solution at room temperature. Phytin was precipitated from a suitable aliquot of the extract with $FeCl_3$ as iron phytate, washed, and converted to the soluble Na-salt by treatment with dilute NaOH. After separation of the $Fe(OH)_3$ precipitate by filtration and washing, phytin-P was determined on an aliquot of the filtrate plus washings made to volume.

*Total acid soluble-P*: A 500 mg sample (if high in inorganic-P) or a 1 g sample (if low in inorganic-P) of the residue from the lipid-P extraction was shaken on a mechanical shaker for 1 hour with 0.75 *N* trichloroacetic acid (TCA). The residue was then separated by centrifuging and washed free of soluble-P with small amounts of the same reagent. Usually 4 or 5 washings were necessary with tissues of high soluble-P contents. Care was taken to retain quantitatively the residue in the centrifuge tube since it was used for the separation of subsequent

[1] Received January 7, 1959.

fractions. Phosphorus was determined on a digested aliquot of the filtered TCA extract. The value obtained was used only to calculate ester-P.

*Inorganic-P*: This fraction was obtained by extracting an aliquot of the TCA extract with isobutyl alcohol which was then determined directly, without digestion, by the isobutyl alcohol colorimetric method (8).

*Ester-P*: Ester-type phosphorus was calculated as the difference between total acid soluble-P and the sum of the values for phytin- and inorganic-P.

*RNA-P*: The tissue residue from the TCA extraction was extracted with 1 *N* perchloric acid at 3° C 3 times at intervals of about 40 hours. Phosphorus was determined in an aliquot of the combined extracts after digestion.

*DNA-P*: The phosphorus extractable from the RNA-P residue above with 0.5 *N* perchloric acid at 70° C.

*Protein-P*: The phosphorus remaining in the residue after separation of DNA-P.

*Total P*: Sum of the separate fractions. When checked, the sum of the fractions agreed well with the values for total phosphorus determined directly.

The data are expressed on the basis of the initial tissue weights when dried at 75° C.

## Results

Germination in the Dark: The concentrations (mg/g) of the various phosphorus fractions in the embryos and seedlings of the 3 cotton varieties are given in table I. Before germination (day 0) the embryos of the 3 cottons differed principally in their phytin-P concentrations and dry weights, being in the order Acala > Paymaster > Deltapine. During germination of all varieties there were successive decreases with time in phytin-P and corresponding increases (except day 1) in the concentration of inorganic-P. Lipid-P increased from day 0 to 2 and then declined but again exceeded its initial concentration by day 6. Ester-P changes were for the most part irregular but tended except in the Acala variety to exceed the original concentration at all times. RNA-, DNA-, and protein-P fractions in general increased in concentration with time of germination.

Changes, during germination, in the amounts ($\mu$g) of the above phosphorus fractions per seedling, using the data for Paymaster in table I, are illustrated in figure 1. To facilitate graphing, RNA- and DNA-P were plotted as the sum instead of separately since they were of similar magnitude.

On the basis of differential solubility in petroleum ether (Pet) and alcohol-benzene azeotrope (AB) there appeared to be at least 2 phospholipid fractions present in the cotton embryos and seedlings (fig 2). At the start of germination these 2 forms were present in a ratio of about 3:1 but after day 2 they tended to become and remain of equal magnitude.

Separation of 6-day-old seedlings of the 3 cotton varieties into cotyledons and hypocotyls plus roots showed that the various phosphorus fractions except phytin-P and RNA-P are fairly evenly distributed between the 2 plant parts (table II). In the varieties tested little or no phytin-P was in the hypocotyl plus roots and the RNA-P content was only about one sixth that of the cotyledons.

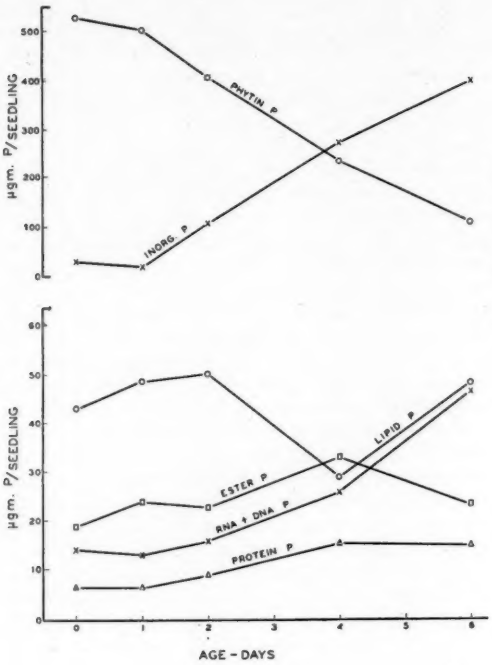
On the basis of mean values of the 3 cottons, phytin-P was found to comprise about 83 %, inorganic-P 4 %, lipid-P 7 %, ester-P 3 %, and RNA-, DNA-, and protein-P each 1 % of the 0-day embryo phosphorous (table III). Germination of the seeds and growth of the seedlings for 6 days in the dark were accompanied by a redistribution of phosphorus among

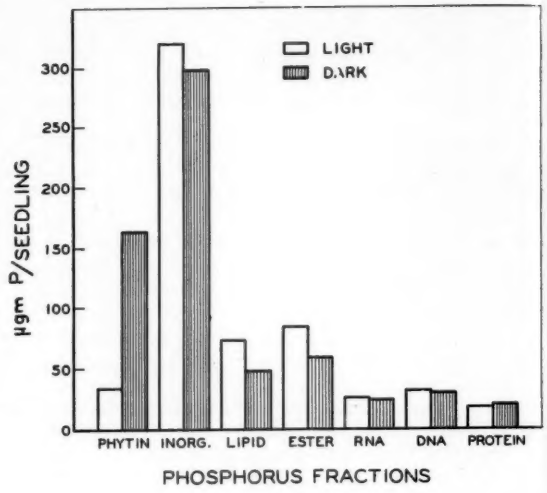
Table I

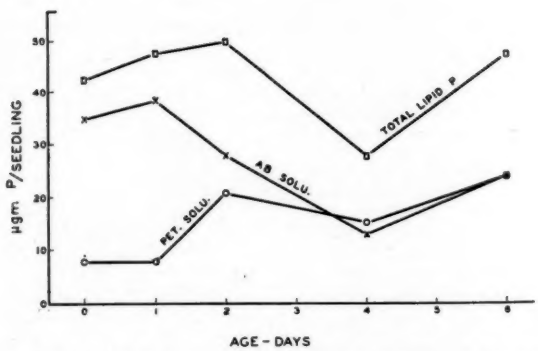
Changes in Dry Weights and the Phosphorus Fractions of Seedlings of Acala, Paymaster and Deltapine Varieties of Cotton During Germination and Growth in the Dark*

| Time | Dry weight | Phytin | Inorg | Total lipid | Ester | RNA | DNA | Protein | Total |
|---|---|---|---|---|---|---|---|---|---|
| *days* | *mg/seedling* | *mg* | *mg* | *mg* | *mg* | *mg* | *mg* | *mg* | *mg* |
| | | | | Acala | | | | | |
| 0 | 82.7 | 9.58 | 0.43 | 0.73 | 0.42 | 0.11 | 0.11 | 0.10 | 11.48 |
| 1 | 82.6 | 9.28 | .37 | .85 | .32 | .09 | .09 | .10 | 11.10 |
| 2 | 80.6 | 8.78 | 1.23 | .89 | .17 | .16 | .13 | .15 | 11.51 |
| 4 | 78.9 | 4.81 | 4.79 | .60 | .48 | .25 | .22 | .31 | 11.46 |
| 6 | 76.9 | 3.49 | 6.26 | .87 | .54 | .39 | .41 | .24 | 12.20 |
| | | | | Paymaster | | | | | |
| 0 | 61.0 | 8.61 | 0.44 | 0.71 | 0.32 | 0.12 | 0.11 | 0.11 | 10.42 |
| 1 | 59.0 | 8.49 | .29 | .81 | .41 | .11 | .11 | .10 | 10.32 |
| 2 | 57.1 | 7.15 | 1.87 | .87 | .40 | .15 | .12 | .16 | 10.72 |
| 4 | 56.8 | 4.00 | 4.77 | .50 | .58 | .25 | .21 | .28 | 10.59 |
| 6 | 56.0 | 1.97 | 7.02 | .85 | .42 | .39 | .44 | .26 | 11.35 |
| | | | | Deltapine | | | | | |
| 0 | 57.3 | 6.49 | 0.29 | 0.71 | 0.14 | 0.11 | 0.11 | 0.11 | 7.96 |
| 1 | 57.1 | 6.24 | .20 | .74 | .18 | .11 | .11 | .11 | 7.69 |
| 2 | 55.8 | 4.53 | 1.54 | .80 | .40 | .18 | .14 | .18 | 7.77 |
| 4 | 54.9 | 2.77 | 3.30 | .56 | .49 | .29 | .23 | .30 | 7.94 |
| 6 | 52.9 | 1.29 | 4.92 | .80 | .44 | .42 | .46 | .23 | 8.56 |

* Phosphorus as mg/g of dry weight.



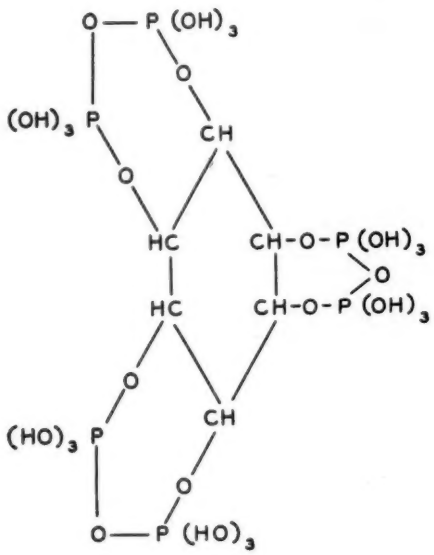




FIG. 1 (*upper left*). Changes in the phosphorus fractions of dark-grown seedlings of Paymaster variety during development.

FIG. 2 (*lower left*). Variations in the petroleum ether (Pet) and alcohol-benzene (AB) soluble lipid-P fractions during development of dark-grown seedlings of Paymaster variety.

FIG. 3 (*upper right*). Influence of light on phosphorus fractions after germination and growth of seedlings for 6 days.

FIG. 4 (*lower right*). Phytic Acid, the principal form of embryo phosphorus.

TABLE II

DISTRIBUTION OF DRY WEIGHT AND PHOSPHORUS FRACTIONS BETWEEN COTYLEDONS AND HYPOCOTYLS PLUS ROOTS OF 6-DAY-OLD SEEDLINGS OF THE THREE COTTON VARIETIES GERMINATED AND GROWN IN THE DARK *

| CONSTITUENTS | ACALA | | PAYMASTER | | DELTAPINE | |
|---|---|---|---|---|---|---|
| | COTYLEDONS | HYPOCOTYL PLUS ROOT | COTYLEDONS | HYPOCOTYL PLUS ROOT | COTYLEDONS | HYPOCOTYL PLUS ROOT |
| | *mg* | *mg* | *mg* | *mg* | *mg* | *mg* |
| Dry weight/ seedling | 46.1 | 32.4 | 29.8 | 27.8 | 28.7 | 27.3 |
| Phosphorus fractions: | | | | | | |
| Phytin | 6.54 | 0.00 | 4.52 | 0.00 | 1.91 | 0.00 |
| Inorganic | 4.24 | 5.31 | 5.15 | 6.13 | 2.68 | 4.53 |
| Lipid A** | .30 | .25 | .28 | .23 | .29 | .21 |
| Lipid B*** | .52 | .51 | .45 | .46 | .47 | .49 |
| Total Lipid | .82 | .76 | .73 | .69 | .76 | .70 |
| Ester | .70 | .92 | .87 | 1.11 | 1.34 | .71 |
| RNA | .61 | .10 | .69 | .07 | .68 | .08 |
| DNA | .42 | .49 | .53 | .46 | .55 | .42 |
| Protein | .28 | .29 | .34 | .32 | .40 | .28 |
| Total | 13.61 | 7.87 | 12.83 | 8.78 | 8.32 | 6.72 |

* Phosphorus as mg/g of dry weight.
** Alcohol-benzene soluble.
*** Petroleum ether soluble.

most of its fractions with phytin- and inorganic-P exhibiting the greatest changes. Lipid-P showed comparatively little net gain and ester-P only slightly more. RNA- and DNA-P, however, each increased more than 3-fold and protein-P, 2-fold.

GERMINATION IN THE LIGHT: Data from the light experiment are given in table IV. Separation of the seedlings into cotyledons and hypocotyls plus roots for analysis in this experiment was done primarily for the purpose of determining whether there was a differential effect of light and darkness on phytin- and RNA-P contents of the hypocotyls plus roots. A comparison of the data in tables II and IV shows there was none; phytin-P was absent and RNA-P remained low and essentially the same in both instances. However, there was an influence of light on certain of the other P-fractions as illustrated in figure 3 in which the mean values of the 3 cotton varieties of table II (dark), calculated on the whole seedling basis, are compared with those of table IV (light). As shown, light germinated seedlings contained substantially less phytin- and more inorganic-, lipid-, and ester-P than the dark germinated ones after 6 days. On the other hand RNA-, DNA-, and protein-P contents were essentially the same under the 2 conditions.

TABLE III

DISTRIBUTION OF PHOSPHORUS AMONG THE PHOSPHORUS FRACTIONS IN EMBRYOS AND 6-DAY-OLD SEEDLINGS *

| PHOSPHORUS FRACTIONS | EMBRYOS | 6-DAY-OLD SEEDLINGS |
|---|---|---|
| | % | % |
| Phytin | 82.9 | 22.0 |
| Inorganic | 3.8 | 56.0 |
| Total Lipid | 7.1 | 7.7 |
| Ester | 3.0 | 4.4 |
| RNA | 1.1 | 3.7 |
| DNA | 1.1 | 4.0 |
| Protein | 1.0 | 2.2 |
| Total | 100.0 | 100.0 |

* Results as mean percentages of the total phosphorus contents of the 3 cotton varieties germinated and grown in the dark.

## DISCUSSION

Phytin is the calcium, magnesium and potassium salt of phytic acid (fig 4) and the foregoing results show that more than 80 % of the embryo phosphorus, irrespective of variety, resides in this single molecular species. Seeds of some other crop plants (8) have equally high phytin-P contents. Another notable feature of phytin, as demonstrated in the current study, is the rapidity with which its phosphorus is liberated, presumably through the action of phytase, during germination. As shown in table I, dephosphorylation was evident as early as day 1 after planting and by day 6 about 60 % of the original phytin-P content had reappeared as inorganic-P (table III). The liberated phosphorus was roughly equally distributed between cotyledons and hypocotyls plus roots of dark-grown seedlings.

Although mobilization of phosphorus from the organic to inorganic-P was the primary reaction in terms of amounts involved, about 10 % of the latter phosphorus was used in the synthesis of additional amounts of organic-P containing compounds originally present in the embryo. For example the RNA- and DNA-P contents of the 6-day-old seedlings were each more than 3 times, and protein-P double that of the embryos. Although there had resulted small net increases in ester- and lipid-P by the 6th day, there was also evidence of small phosphorus loss earlier from these 2 sources. There was a substantial decrease in amounts per seedling of total lipid-P between days 2 and 4, and ester-P between days 4 and 6 (figs 1, 2),

Utilization of phosphorus from the latter source is not surprising since this group of compounds comprise, among others, adenosine triphosphate, uridine triphosphate and other nucleotides with energy-rich phosphate bonds that mediate energy transfer within the cell. Since little is known about the phospholipid metabolism of plants it is difficult to determine the significance of the loss and subsequent recovery of the lipid-P. Actually, as shown in figure 2, the alcohol-benzene (AB) soluble lipid-P fraction declined after day 1 but this loss is accounted for by a corresponding increase in the fraction soluble in petroleum ether (Pet). Thereafter changes in the 2 fractions were similar. It is not known at this time whether these 2 fractions represent respectively cephalin and lecithin, the dominant phospholipids of the great majority of plant tissues. Lecithin often constitutes about one third and cephalin two thirds of the total plant phospholipids. In the present instance this proportion, on the basis of lipid-P, was approached in the respective Pet and AB soluble extracts of the embryos but not of the seedlings.

Separation of 6-day-old dark-grown seedlings into cotyledons and hypocotyls plus roots for analysis showed some differences in the phosphorus metabolism of the 2 organs. The absence of phytin-P in the 6-day-old hypocotyls plus roots (also in those from a 3-day germination through the data are not reported) suggest that it is not translocated from the cotyledons as such. Instead, dephosphorylation occurs principally in the cotyledons and substantial amounts of the resulting inorganic-P then move into the hypocotyls plus roots. How much of the translocated phosphorus was used in the synthesis of the other organic fractions is beyond the scope of this paper. The results showed that the organic-P compounds, with the exception of RNA-P, accumulated in the hypocotyls plus roots in amounts similar to those found in the cotyledons. Inasmuch as RNA is generally regarded as a template for the incorporation of amino acids into protein (2), this difference may be a reflection of characteristic differences in protein levels of the 2 organs, being slightly higher in the cotyledons. Regardless of the site of synthesis, it is of interest that the hypocotyls plus roots of the 3 varieties contained only about one-sixth to one fifth as much RNA- as DNA-P whereas they occurred in the cotyledons in nearly equal amounts.

In the experiment in which germination was conducted in sunlight, the seedlings, as in the dark experiment, started emerging the 4th day after planting. Thus it is estimated that the light-germinated seedlings probably received a total of 24 to 30 hours of light for photosynthesis. It was shown that light (table IV) under these conditions caused (a) a further increase in rate of phytin hydrolysis, with a concomitant increase in inorganic-P, and (b) the synthesis of additional amounts of ester- and lipid-P compounds, compared to germination in the dark (table II). The other phosphorus compounds, RNA, DNA, and protein, were essentially unaffected. The increase in ester-P appears to be logical since phosphoglyceric acid is known to be the major initial product of photosynthesis followed by the phosphorylated sugars and still other ester-P compounds. Though less is known of the influence of light on lipid metabolism, it is pertinent that Smith (10) also reported a rapid synthesis of ether-soluble phosphorus compounds and lipoidal material when etiolated barley seedlings were illuminated, and that phospholipids are among the components of chloroplastic material.

Turning to differences among the 3 cotton varieties in their mobilization and utilization of phosphorus, it was noted that phosphorus content and metabolism were roughly correlated with embryo and seedling weight. That is, the heavier the embryo the more of

TABLE IV

DRY WEIGHTS AND PHOSPHORUS FRACTIONS OF COTYLEDONS AND HYPOCOTYL PLUS ROOTS OF 6-DAY-OLD SEEDLINGS OF ACALA, PAYMASTER AND DELTAPINE GERMINATED AND GROWN IN THE LIGHT *

| CONSTITUENTS | ACALA | | PAYMASTER | | DELTAPINE | |
|---|---|---|---|---|---|---|
| | COTYLEDONS | HYPOCOTYL PLUS ROOT | COTYLEDONS | HYPOCOTYL PLUS ROOT | COTYLEDONS | HYPOCOTYL PLUS ROOT |
| | *mg* | *mg* | *mg* | *mg* | *mg* | *mg* |
| Dry weight/ seedling | 61.4 | 18.8 | 41.2 | 17.5 | 38.5 | 17.8 |
| Phosphorus fractions: | | | | | | |
| Phytin | 1.00 | 0.00 | 0.59 | 0.00 | 0.46 | 0.00 |
| Inorganic | 6.34 | 5.29 | 5.36 | 3.79 | 3.07 | 3.44 |
| Lipid A** | .63 | .31 | .69 | .31 | .57 | .30 |
| Lipid B*** | .66 | .48 | .57 | .46 | .52 | .53 |
| Total Lipid | 1.29 | .79 | 1.26 | .77 | 1.09 | .83 |
| Ester | 1.80 | .68 | 1.53 | .31 | 1.20 | .71 |
| RNA | .56 | .09 | .55 | .08 | .54 | .08 |
| DNA | .47 | .43 | .49 | .44 | .50 | .47 |
| Protein | .29 | .25 | .28 | .29 | .30 | .28 |
| Total | 11.75 | 7.53 | 10.06 | 5.68 | 7.16 | 5.81 |

* Phosphorus as mg/g of dry weight.
** Alcohol-benzene soluble.
*** Petroleum ether soluble.

each phosphorus fraction was found. However phytin-P, and consequently total-P, was found to vary significantly with variety even when the results were expressed on a concentration basis (mg/g). This fraction (table I) was always (before and during germination) present in higher concentrations in the late (Acala) than in the 2 early maturing varieties (Paymaster and Deltapine). The significance of this, if any, as a biochemical distinction between earliness and lateness in cottons will, of course, have to be determined by further study. This deficit of phosphorus was certainly of no disadvantage in early development as all 3 varieties were observed to grow equally well (hypocotyl plus root elongation data obtained but not reported).

Although earliness in cotton may be influenced by environmental factors it is primarily under genetic control. Of the 7 phosphorus containing compounds reported in this study, DNA is the 1 most frequently found to be associated with heritable characters and is often referred to as the "carrier" of heredity. In this study the concentrations of DNA in the 3 varieties, as indicated by its phosphorus content, were remarkably similar before and during germination. No differences should be expected since the DNA content of cells, except when dividing, tends to be constant (6). Differences in DNA, which have been found to characterize the source, are usually found in the nucleotide composition of the DNA molecule itself: DNA acids of different species of organisms have different nucleotide compositions (6).

## Summary

Successive changes in the amounts and concentrations of the phosphorus containing compounds of 2 early- and 1 late-maturing varieties of cotton during a 6-day germination period were followed analytically.

The dominant process of phosphorus metabolism in the germinating seed was the dephosphorylation of phytin with the simultaneous accumulation of relatively large amounts of inorganic-P. However, germination was also marked by the synthesis and accumulation of additional amounts (beyond those of the embryos) of RNA-, DNA-, protein-, ester- and lipid-P compounds.

Light, compared to darkness, increased the rate of phytin hydrolysis and the synthesis of lipid- and ester-P compounds during germination and early seedling growth but had no appreciable effect on the other phosphorus compounds.

In the partition of phosphorus, the hypocotyls plus roots of 6-day-old seedlings differed mainly from the corresponding cotyledons in that the former contained little or no phytin- and only about one sixth as much RNA-P.

Varietal difference was limited to phytin-P which was present in the embryos and seedlings of the late variety in substantially greater amounts and concentrations than in the 2 early-maturing cottons.

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# WINTER FREEZING IN RELATION TO THE RISE OF SAP IN TALL TREES[1]

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The cohesion theory of Dixon and Joly (4) and Askenasy (1) has generally gained acceptance as the most likely explanation of how water rises to the top of tall trees (12). It may be taken as reasonably well established that in the summertime there are continuous water columns in the xylem, and experimental evidence that cohesive tensions of sufficient magnitude may develop in tall trees and lianas in a period of transpiration, has been furnished by experiments of potometer type (14, 21, 22, 23, 24, 25).

One may assume that leaf shedding does not per se break any such tension, as it has been shown that the walls of the vessels and tracheids will not let through a gas-water interface (29). With exception of the grapevine, and probably some other species which have air-filled vessels in the winter (28), it is known that the water content at all levels in the xylem of several species of trees remains as high or nearly as high in winter as in summer (7, 9, 15, 16, 30).

When water freezes, dissolved gas is forced out as bubbles. This happens whether water freezes in bulk or in glass capillaries (27). It has been shown that the sap of the grapevine is fully saturated with dissolved atmospheric nitrogen (28), and the same very likely holds also for other species. One must, therefore, assume that gas bubbles are formed whenever freezing takes place in sap-filled vessels and tracheids, and a great many of these would hence become emptied and eliminated for the transport system, were the sap under tension at the time of thawing. The only mechanism which might conceivably repair such breaks, would be a sap pressure high enough to redissolve the gas. In the case of the grapevine, which empties its vessels during the winter, the root pressure in the springtime is so high that it may readily refill the conducting system (28). But such pressures are at present known only in a few hardwood species (3, 8, 13, 17, 32).

Sub-zero temperatures with freezing of the sap, therefore, are difficult to fit into the cohesion theory, but this difficulty will not arise if the sap remains supercooled throughout the winter. A few degrees of supercooling has been produced experimentally in different species of trees, but this was observed for a brief period only (20).

In the present investigation the status of the sap has been studied in various species of trees at subzero temperatures.

[1] Received January 9, 1959.

## MATERIALS AND METHODS

The following species have been used for the observations and experiments: Spruce (*Picea excelsa* (Lam.) Link.), pine (*Pinus silvestris* L.), linden (*Tilia cordata* Mill.), birch (*Betula odorata* Bechst.), maple (*Acer platanoides* L.), ash (*Fraxinus excelsior* L.), alder (*Alnus incana* (L.) Moench.), asp (*Populus tremula* L.) and willow (*Salix sp.*).

BUBBLE FORMATION WHEN WATER FREEZES IN CAPILLARIES: Water-filled glass capillaries, about 30 $\mu$ in diameter, were frozen at $-8°$ C and examined under the microscope. A string of gas bubbles could be seen in the center of the ice, and after thawing, the bubbles persisted in the water for several days. This also happened when both ends of the capillary were sealed with grease, so no air could be drawn in from the outside.

Other glass capillaries (0.03 to 0.3 mm in diameter), bent to a Z shape and filled with water, were spun in the centrifuge by the method of Briggs (2). The pressure at the center was calculated to be $-1.8$ atmospheres. Freezing of the water by applying dry ice to the center of the spinning capillary (fig 1) resulted in immediate breaking of the water column. These experiments show that gas bubbles are formed and cohesion is broken when water freezes in glass capillaries of vascular or tracheidal dimensions.

Pieces of fresh spruce wood were put in a dish, covered with kerosene and observed with a microscope. The tracheids were water-filled and without bubbles. The dish was placed in a cold room, and the pieces were frozen at $-8°$ C. Upon thawing, gas bubbles could be seen in many of the tracheids, although not in all.

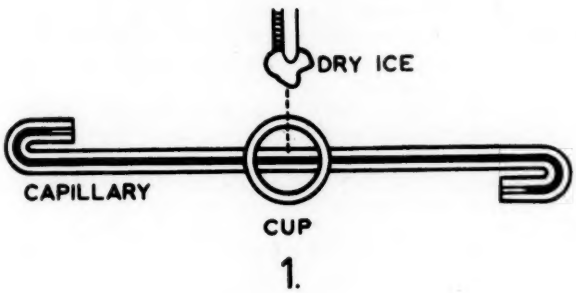
DEMONSTRATION OF ICE IN TWIGS: During subzero temperatures twigs of many trees become brittle, and several authors have observed ice microscopically in different parts of living plants in the winter (19, 31, 33, 34); but one may ask to what extent the manipulation may possibly have caused a supercooled plant to freeze.

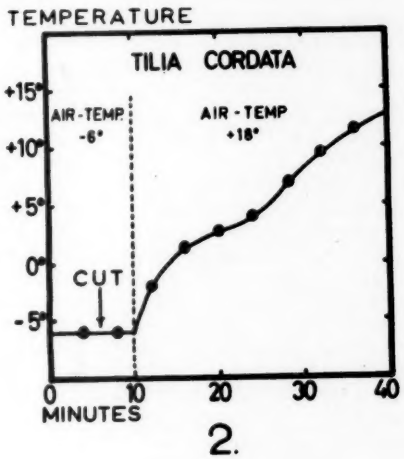
When ice is present in a plant it can easily be demonstrated by the heat of fusion. Many experiments were performed in the following manner. A thermocouple, covered by cotton, was placed on a twig of linden. The temperature was $-6°$ C. When the twig was severed from the tree near the thermocouple, the temperature remained unchanged, nor did it rise when the cut end was seeded with snow. At room temperature a typical melting curve was ob-

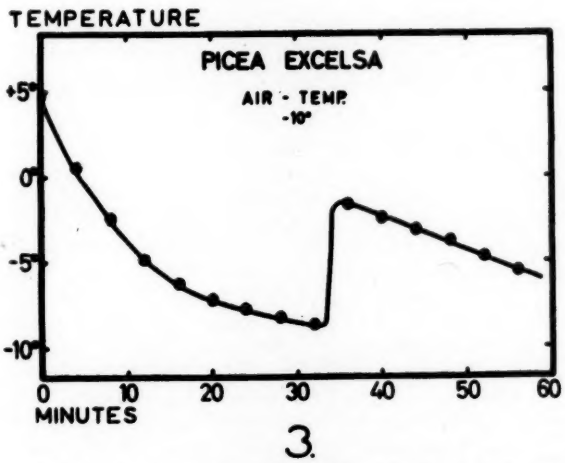
served, showing that the twig was already frozen while on the tree (fig 2).

EXTENT OF SUPERCOOLING: Fresh twigs of spruce and pine (1 × 10 cm) were kept for several hours in a cold room at 5° C. Thermocouples were placed on the outside of the bark underneath a piece of cotton and in bore holes in the wood. Both ends of the twigs were greased to prevent drying and to avoid accidental seeding with ice in the tracheids. The twigs were transferred to −10° C. Supercooling was regularly observed, but spontaneous freezing took place within one half hour. Lowest supercooling temperature observed was −8.7° C (fig 3).

Branches of spruce, pine, birch, maple, ash, alder, asp and willow were sawed off at ground level or near the top of trees that were 12 to 14 meters high. The temperature in the wood was measured immediately with a thermocouple in bore holes and found to be the same as the air, i.e. −7° C. The wood around the thermocouple was then crushed with a pair of tongs and bent and broken, but the temperature remained unchanged. The same results were obtained



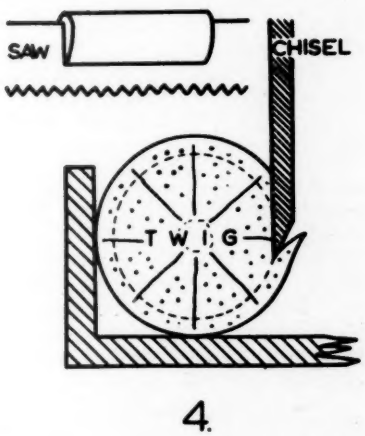




FIG. 1. Z-bent capillary mounted horizontally in the centrifuge (Briggs 1950). Center of the capillary at the spinning axis. Dry ice may be dropped in the cup while spinning.

FIG. 2. Temperature curve showing heat of fusion in a twig of linden, which was cut off and warmed to room temperature.

FIG. 3. Temperature curve showing supercooling of twig of spruce when transferred to a −10° C cold room.

FIG. 4. Twig end with bark shaved off. Saw with depth guide. Pieces used for the calorimetric measurements were broken off with a chisel.

when the thermocouple was put into the trunk of small trees which were similarly crushed and bent. This shows that the trees at all levels were frozen and not supercooled.

QUANTITATIVE DETERMINATIONS OF ICE IN THE XYLEM: Branches from different levels above ground or small stems of spruce, birch and maple were taken in March and brought without thawing directly to the cold room.

The amount of ice in the xylem was measured by a calorimetric method introduced by Müller-Thurgau (11, 18, 20, 26). A thermos bottle with 250 ml distilled water was used as calorimeter. A thermometer which could be read within ±0.02° C was inserted through the paraffined cork stopper. The heat capacity of the thermos with water was determined by dropping into it pieces of ice of known weight and temperature. Eight such measurements agreed within ±1.5 %.

After the branches or stems (3 to 4 cm thick) had remained at least 24 hours in the cold room at a desired sub-zero temperature, the following operations were conducted, taking care not to heat the samples. The twig ends were discarded, because of possible drying, and the bark trimmed off. Shavings, 5 to 6 cm long, were cut with a chisel from the outer 3 to 4 mm of the functional xylem (fig 4). About 20 g of the shavings were put into a wide-mouthed plastic funnel. The calorimeter, containing water of near 20° C, was brought into the cold room, received the shavings and was then immediately returned to room temperature. In one half minute, or less, the temperature became steady 2 to 3 degrees lower, and was read. The shavings were placed in an oven of 110° C for about 24 hours to obtain dry weight and total water content.

The amount of ice in the sample was calculated from the equation:

$$M_i = \frac{C(T_2 - T_3) - (S_d M_d + S_w M_w)(T_3 - T_1)}{H_i - (S_w - S_i)(T_o - T_1)}$$

where C is heat capacity of the calorimeter; $M_d$, dry weight of the sample; $M_w$, total water content in the sample; $M_i$, amount of ice in the sample; $S_d$, specific heat of wood; $S_w$, specific heat of water = 1.0[2]; $S_i$, specific heat of ice = 0.5; $H_i$, heat of fusion for ice = 79.9; $T_o$, freezing point of the sap in the samples; $T_1$, initial temperature of the sample; $T_2$, initial temperature of the calorimeter; $T_3$, final temperature of sample in calorimeter.

The specific heat of wood ($S_d$) does not vary measurably with species, habitat or anatomical part, but has been corrected for temperature according to the equation

$$S_d = 0.266 + 0.00116 \frac{T_3 + T_1}{2} \quad \text{(Dunlap 1912)}$$

[2] The specific heat of the unfrozen water very likely lies between that of ice (0.5) and that of free water (1.0). If it were 0.7, we get a decrease of about 10 % in the calculated amount of unfrozen water.

We have here calculated with a mean value for $S_d$ = 0.27. The freezing point of the sap has been taken as 0° C. Even if it were −2° C, it would give no more than 3 % increase in the calculated amount of unfrozen water.

## RESULTS

The amount of unfrozen water has been calculated in relation to the dry weight of the samples (figs. 5, 6 and 7). It will be seen that it constitutes some 30 to 40 % of the dry weight and decreases slightly with lowering of the temperature. This relation is independent of the total water content of the sample and shows that the unfrozen water is essentially that which is bound to the cellulose walls. About one tenth of the unfrozen water can be accounted for by the increased osmotic concentration of the sap as water freezes out.

The water content of a colloidal system depends upon the relative humidity, but is influenced very little by temperature (10). In the presence of ice, however, the relative humidity decreases with temperature. Our data for unfrozen water at sub-zero temperatures can, therefore, be compared with data for water content in white spruce at different relative humidities (6). As will be seen from figure 8, the curves are very similar, although showing slight species differences.

From these results we conclude that in winter time practically all sap within vessels and tracheids of the trees investigated freezes, forcing the dissolved gases out as bubbles at all levels.

## DISCUSSION

It has been demonstrated in the present investigation that the free sap in the xylem of several species of trees freezes during the winter. All or the main part of the sap left unfrozen is bound to the cellulose in the wood. It is known that when water freezes in capillaries, dissolved gases are forced out as bubbles. The sap in the vessels of grapevine has been found to be fully saturated with atmospheric nitrogen (12 to 13 mm³/ml) and more than half saturated with atmospheric oxygen (4 to 5 mm³/ml) (28). Very likely, this holds also for other species. If all this gas were forced out by freezing of the sap, it would amount to about 2 % of the ice volume.

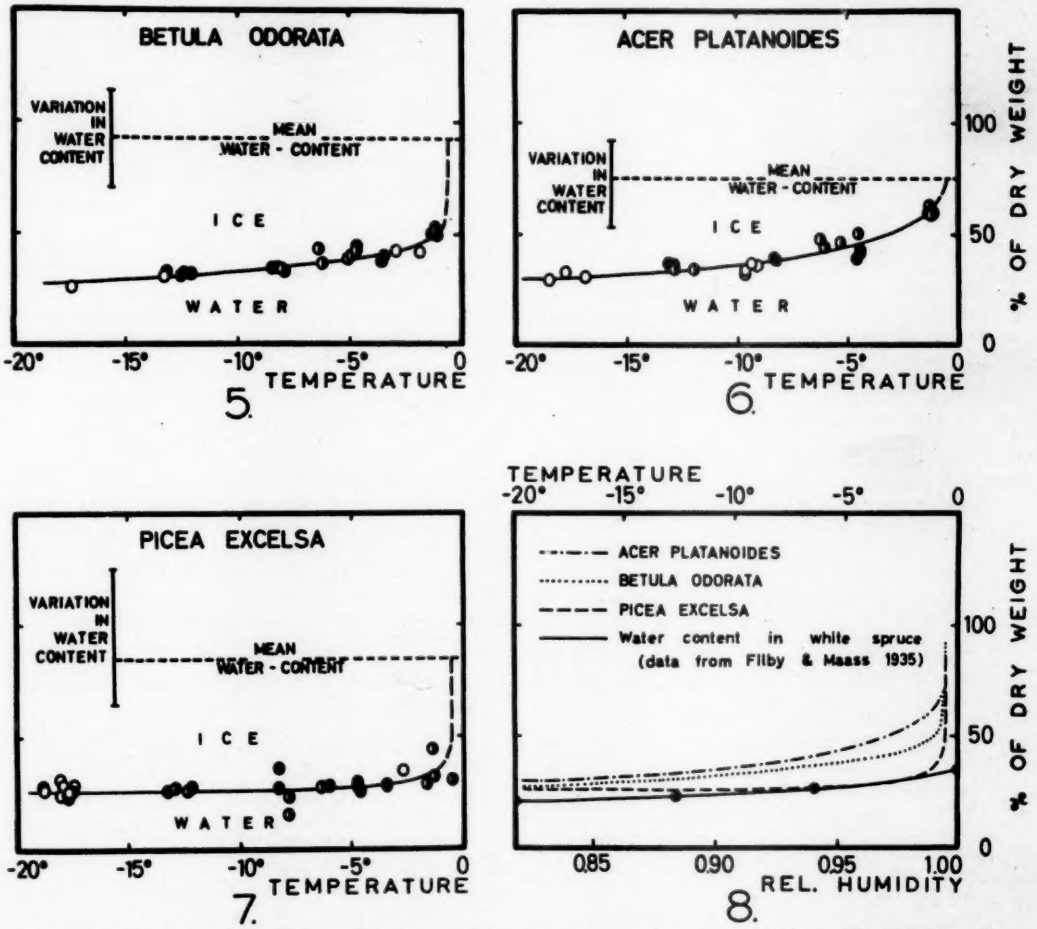
Were the sap under tension during freezing or thawing, the gas bubbles would expand and fill the units in which they were formed, and if the gas evolution were evenly distributed, bubble formation would destroy the cohesion in all tracheids and vessels. It was shown in our spruce experiments, however, that some tracheids did not contain bubbles after thawing. Evidently, therefore, some diffusion may take place through the walls. Migration of gas over great distances is unlikely, however, because supercooling will generally precede freezing, which then becomes a relatively even and rapid process.

With repeated freezing and thawing throughout the winter, one would expect an increasing number of elements to be set out of function by gas bubbles, but regular winter freezing is neither fatal nor even injurious to trees in cold climates. Therefore, one may assume that some mechanism exists which is capable of repairing gas breaks, and the only known mechanism which could accomplish this is a high hydrostatic pressure in the xylem. However, high stem pressures in the spring are known for a few hardwood species only, and the question still remains open: How are the gas breaks repaired? Accurate measurements of hydrostatic pressure gradients can give a clue to the solution of this problem.

## Summary

The status of sap in the xylem of tall trees during sub-zero temperatures has been investigated. Preliminary experiments showed that when water was frozen in fine glass capillaries, gas bubbles always separated out. By spinning the capillary in a centrifuge, the water was put under tension, and freezing resulted in breaking the cohesion. Gas bubbles were also formed in many tracheids of spruce wood when water-filled pieces were frozen and thawed under kerosene.

Twigs and stems of several species of trees were investigated at freezing temperatures. When crushed, broken and seeded with snow, their tempera-




Figs. 5 to 7. Percent unfrozen water in xylem at various temperatures. Open circles: Material taken frozen in winter and transferred to cold room. Filled circles: Material taken in summer and transferred directly to cold room. Half filled circles: Material taken in summer, transferred to cold room after a few days at 5° C.

Fig. 8. Unfrozen water in spruce, birch and maple at sub-zero temperatures compared to water content in wood of white spruce at various relative humidities (Filby and Maass 1935). Abscissas give sub-zero temperatures and the equivalent relative humidities.

ture did not change, and at room temperature a typical melting curve was observed. It was concluded that the sap of the trees examined was not supercooled, but frozen. A slight degree of supercooling in cut twigs of spruce and pine could readily be produced experimentally, but persisted for a short time only.

The amount of unfrozen water in the xylem of spruce, birch and maple was measured calorimetrically and amounted to a constant fraction (30 to 40 %) of the dry weight. It decreased with temperature, but was independent of the total water content. This shows that the unfrozen water was bound to the wood leaving all sap within tracheids and vessels frozen. Freezing causes release of the dissolved gas as bubbles in the conducting elements. If the sap is being transported by cohesive lift, a mechanism must exist which can mend such breaks, but observational clues are lacking as to how this comes about.

I am much indebted to Professor P. F. Scholander for his great interest and inspiring help through all stages of the work. My thanks are due to the staff and students of the Institute of Zoophysiology, University of Oslo for their interest and assistance in the experiments.

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# NEWS AND NOTES

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, *Midwest Section*: The fifth Annual Meeting of the Midwest Section was held at Purdue University on June 8 and 9, 1959. Approximately 80 members attended the sessions, as well as 30 visitors from among several departments on the Purdue campus. The program on Monday morning included 17 research reports. These were followed, in the afternoon, by a symposium on "Some Effects of Light on Plants", during which talks were presented by Dr. Alice Withrow on "Kinetics of Photomorphogenesis," Dr. H. W. Siegelman on "Photocontrol of Anthocyanin Synthesis," Dr. S. Gordon on "Phosphorylation and Photomorphogenesis" and Dr. J. W. Hastings on "Clocks and Metabolism." Contributed papers were also presented on Tuesday, as well as organized discussions of "Controlled Climate Facilities," "Senescence and Dormancy," and "Physiology of Cell Walls."

The annual dinner featured a somewhat scholarly review of the parentage and origins of John Purdue and Plant Physiology in the inimitable style of the chairman, Dr. Harry Beevers. To round out an entertaining program, Dr. Carl Leopold presented a sensitive and professional rendition of old- and near-English folk songs and ballads, including some delicate and touching ones by Tom Lehrer. An open house in the plant physiology laboratories of the Life Sciences Building and the Horticulture Building completed the organized phases of the meetings.

Officers elected for 1960 were: Dr. Robert Burris, Chairman; Dr. Robert Bandurski, Vice-Chairman; Dr. Carlos Miller, Secretary-Treasurer; and Dr. Harry Beevers, Representative to the American Society of Plant Physiologists.—ALFRED S. SUSSMAN, *Secretary-Treasurer, Midwest Section.*

INTERNATIONAL SYMPOSIUM OF AGRICULTURAL CHEMISTRY: The Organization Committee for the Third International Symposium of Agricultural Chemistry to be held at Seville, October 17-22, 1960, invites contributions on "Problems of Nitrogen in Agriculture." Interested participants should communicate with the Organization Committee at the Institute of Agricultural Chemistry of the University of Pisa, Via S. Michele degli Scalzi 2 (Phone 23.312) by August 30, 1959 and submit titles and brief resumes of their contributions.

AN AMENDMENT TO THE BY-LAWS OF THE CONSTITUTION OF THE AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS: The proposed amendment is to change the monetary award of the Stephen Hales prize from $100 to $500. The proposal involves the following changes in Section 9-A of the By-Laws:

After "shall make from the available funds" insert "from income of the endowment and general funds of the Society" and change $100 to $500 throughout the Section.

This action has been approved by the unanimous vote of the Executive Committee and has been presented to the membership of the Society at the annual meeting held in Montreal, Canada on August 26, 1959. It will become effective one year from the date of publication in conformity with the By-Laws. —STERLING B. HENDRICKS, *President, ASPP.*

# AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

**Organized in 1924 for the advancement of Plant Physiology as a pure and applied field of botanical science**

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Any one actively interested in Plant Physiology is eligible for membership in the Society. Candidates for membership must be recommended by two members, and approved by executive committee, or by the vote of the Society at some annual or special meeting.

The payment of the annual dues to the Society entitles the members to receive the official journal, Plant Physiology. They will receive in addition the regular bulletins and announcements sent out periodically by the secretary. Members are invited to take a personal interest in the growth and development of the Society.

Correspondence concerning membership and its privileges, dues, change-of-address, etc., should be addressed to the Executive Secretary-Treasurer, Dr. G. R. Noggle, ASPP, Box 2665, University Station, Gainesville, Florida. Business correspondence regarding subscriptions, back numbers, change-of-address of subscribers, etc., may be addressed to Plant Physiology, Box 749, Lancaster, Pa., or to the Executive Secretary-Treasurer at the address above. All matters pertaining to nominations, elections, programs, and meetings of the society should be addressed to the Secretary, Dr. Walter D. Bonner, Jr., Cornell University, Ithaca, New York. Correspondence with reference to editorial matters should be addressed to the Editor, Dr. Allan H. Brown, Department of Botany, University of Minnesota, Minneapolis 14, Minnesota.